Biosynthesis of aminocoumarin antibiotics in *Streptomyces*: Investigations on the regulation of novobiocin production

Biosynthese von Aminocoumarin-Antibiotika in *Streptomyces*: Untersuchungen zur Regulation der Novobiocinproduktion

DISSERTATION

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Dangel, V., Westrich, L., Gust, B. & Heide, L. Improved novobiocin biosynthesis in *Streptomyces coelicolor* M512 by regulation threw tetracycline-controllable promoters. (in preparation)

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Oral presentations


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<tr>
<td>°C</td>
<td>degree celsius</td>
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<tr>
<td>µ</td>
<td>micro</td>
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<tr>
<td>aa</td>
<td>amino acids</td>
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<td>aac(3)IV</td>
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<td>sodium hydroxide</td>
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<tr>
<td>neo</td>
<td>neomycin/kanamycin resistance gene</td>
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nt  nucleotide
OD$_{600}$  optical density at 600 nm
ORF  open reading frame
oriT  origin of transfer from RK2
p  pico
PCR  polymerase chain reaction
PEG  polyethylene glycol
R  resistant
RBS  ribosome binding site
Ring A  3-dimethylallyl-4-hydroxybenzoic acid
RNA  ribonucleic acid
RNase  ribonuclease
RP  reverse phase
rpm  rotation per minute
RT  Reverse transcriptase
s  second
s.  see
S.  Streptomyces
S. roseochromogenes  S. roseochromogenes var. oscitans
ssDNA  single-stranded DNA
TEMED  N,N,N',N'-tetramethylethylenediamine
TES  N-Tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid
Thio  thioestrepton
Tris  2-amino-2-(hydroxymethyl)-1,3-propanediol
Tris-maleate  Tris-(hydroxymethyl)-aminomethane-maleate
Topo  topoisomerase
U  unit
UV  ultraviolet
WT  wild-type
×g  ground acceleration
X-gal  5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
SUMMARY

The aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A₁ are produced in different Streptomyces strains and are potent inhibitors of DNA gyrase. Cloning and sequencing of the corresponding biosynthetic gene clusters allowed detailed investigations of their biosynthetic pathways as well as the generation of novel antibiotics by metabolic engineering, chemo-enzymatic synthesis and precursor-directed biosynthesis. On the other hand, only limited knowledge is available about the regulation of the biosynthesis of the aminocoumarin antibiotics.

The biosynthetic gene cluster of novobiocin, clorobiocin and coumermycin A₁ each contains two putative regulatory genes with high similarity in between the clusters, i.e. novG/cloG/couG and novE/cloE/couE. The function of NovG as a DNA binding protein and positive regulator of novobiocin biosynthesis has been established previously. In the first part of this thesis, functional proof for the role of novE as a positive regulator of novobiocin biosynthesis is provided. Overexpression of novE, using a replicative shuttle vector in S. coelicolor strains carrying the intact novobiocin cluster has been shown to lead to almost two-fold overproduction of novobiocin, suggesting that novobiocin production is limited by the availability of NovE protein. In contrast, a novE-defective mutant, generated by an in-frame deletion in this study, produced only 0.7 % of the novobiocin amount formed by an S. coelicolor strain harboring the intact novobiocin cluster. Novobiocin production in this ΔnovE mutant could be restored by introduction of an intact copy of novE, but also by overexpression of the regulatory gene novG.

NovE was expressed in E. coli and purified. However, in contrast to NovG, no DNA binding properties could be shown for NovE. The following RT-PCR experiments showed that at least some novG transcription can occur in the absence of NovE, and that novE transcription can occur in the absence of NovG. Correspondingly, overexpression of novG under control of its own promoter stimulated novobiocin production even in a novE-defective mutant.

Another part of this thesis focuses on the determination of promoter regions within the novobiocin biosynthetic gene cluster. For this purpose Ω (omega) interposons, i.e.
DNA fragments containing an antibiotic resistance marker flanked by short inverted repeats containing termination signals for transcription, were introduced into genes downstream of putative promoter regions, i.e. into novE, novF, novG, novH, novO, novP, novQ and novS, resulting in termination of mRNA synthesis at the place of insertion. Transcription is re-initiated at the next active promoter-sequence downstream of the Ω insertion. RT-PCR analysis of the generated mutants showed that the novobiocin biosynthetic gene cluster contains, in addition to previously identified promoter regions upstream of novE and gyrB<sup>R</sup>, six further promoter regions situated upstream of novF, novG, novH, novO, novP and novQ.

In order to confirm the importance of the promoter regions identified upstream of novO, novP and novQ, quantitative RT-PCR experiments were carried out to quantify transcription of novH, novP and novQ in the ΩnovH mutant in comparison to S.coelicolor strains containing the intact novobiocin cluster. The results of these investigations clearly showed that the Ω insertion into novH resulted not only in an almost complete loss of novH transcription (< 1 %), but additionally in a very strong reduction of transcription of novP and novQ (<3 %). This finding strongly suggests that transcription of novO, novP and novQ (and of the genes located downstream thereof) is mainly controlled by the novH promoter initiating a large transcript of at least 18 kb, i.e. from novH to novW. Furthermore, quantitative RT-PCR was used for investigations of the interplay of the two positive regulators novE and novG, as well as on their influence on the novobiocin biosynthetic genes. These investigations showed that both novE and novG act as transcriptional activators of the genes of novobiocin biosynthesis by initiating transcription from the novH-promoter. novE and novG act in a cascade-like reaction mechanism, i.e. novE positively regulates transcription of novG and NovG regulates transcription of all genes from the novH promoter by binding to a well-defined inverted repeat sequence in the intergenic region between novG and novH.

Based on the results presented above, the final part of this thesis deals with the uncoupling of novobiocin production from its natural regulation cascade by replacing the entire novEFG-region, including the promoter region upstream of novH, by a strong inducible promoter. For this purpose, the tetracycline-controllable promoter 830 (tcp<sub>830</sub>) was used. It has been shown previously, by using luxAB genes expressing luciferase as a reporter system, that induction factors of up to 270 could
be obtained for tcp830 by induction with anhydrotetracycline. HPLC analysis of novobiocin production in the resulting mutants showed that an induced tcp830 promoter is sufficient to cause transcription of the genes from novH to novW, i.e. of a polycistronic mRNA of >18,000 nt, resulting in a two-fold overproduction of novobiocin in comparison to strains containing the unmodified novobiocin gene cluster. Therefore, regulation of novobiocin production by tcp830 has been confirmed as a strategy to uncouple novobiocin production from its natural regulation and notably as a further, newly discovered tool to enhance antibiotic production.


Zur Bestätigung der Rolle der Promotorregionen upstream von novO, novP und novQ, wurden quantitative RT-PCR (qRT-PCR) Experimente durchgeführt um die Transkription von novH, novP und novQ in der Ω-novH-Mutanten im Vergleich zu Streptomyces coelicolor-Stämmen mit intaktem Novobiocincluster zu quantifizieren. Die Ergebnisse dieser Untersuchungen zeigten eindeutig, dass die Ω-Insertion in novH nicht nur zu einem nahezu vollständigen Ausfall der novH-Transkription (<1 %), sondern auch zu einer sehr starken Reduktion der Transkription von novP und novQ (<3 %) führt. Diese Ergebnisse machen wahrscheinlich, dass die Transkription von novO, novP und novQ sowie der downstream davon liegenden Gene hauptsächlich über den novH-Promotor kontrolliert wird, von dem ein mindestens 18 kb großes Transkript, das heißt von novH bis novW, gebildet wird. Außerdem wurde mittels qRT-PCR das Zusammenspiel der beiden positiven Regulatoren novE und novG, sowie deren Einfluss auf die Novobiocin-Biosynthesegene untersucht. In diesen Untersuchungen wurden novE und novG als Transkriptionsaktivatoren der Novobiocinproduction bestätigt, welche in einer Regulationskaskade die Transkription vom novH-Promotor aus initiieren. Offenbar aktiviert novE die Transkription von novG und NovG reguliert über den novH-Promotor die Transkription aller Gene durch Bindung an eine definierte Sequenz im intergenischen Bereich zwischen novG und novH.
I. INTRODUCTION

I.1. *Streptomyces* – the largest antibiotic-producing genus

*Streptomyces* spp. are Gram-positive, filamentous, soil bacteria and ubiquitous in nature. They belong to the order Actinomycetales. Remarkable features of *Streptomyces* are the high G+C ratio of their DNA (> 70%) and their large chromosome, which consists of 8-10 Mb (Bentley *et al.*, 2002; Bibb *et al.*, 1984; Ikeda & Nakagawa, 2003).

Furthermore, they are remarkable in terms of their morphological and metabolic differentiation phenomena (Hopwood, 1999). During their complex life cycle, *Streptomyces* differentiate into at least three distinct cell types: substrate hyphae, aerial hyphae and spores. Germination of spores is the first step in the development of *Streptomyces*, giving rise to a basal or substrate mycelium, which develops in close contact with the nutritive substrate. In response to poorly identified signals emanating from the environment and from cellular metabolism, the basal mycelium develop aerial hyphae, which ramify and differentiate into spores (Chater, 1998; Chater & Horinouchi, 2003). The passage from one mycelial state to the other is characterised by a pause in growth and requires the expression of numerous genes specific to the aerial mycelium.

This morphological differentiation is accompanied by metabolic differentiation. Generally, *Streptomyces* spp. produce a large number of very diverse secondary metabolites during the later stages of this development. Genes responsible for the synthesis of specific secondary metabolism products are clustered on the chromosome. These clusters may contain all biosynthetic, regulatory and resistance genes required for production of antibiotics. Notably, over two thirds of the clinically useful antibiotics of natural origin are produced by Streptomycetes (Kieser *et al.*, 2000). Till now, over 3000 biological active compounds have been isolated from Streptomycetes (including important antibiotics like tetracyclins, vancomycin and erythromycin) (Watve *et al.*, 2001). Further bioactive compounds produced by streptomycetes are used as antitumoural, antifungal and antiviral agents or as herbicides.
I.2. Regulation and overproduction of antibiotics production in *Streptomyces*

Antibiotics represent the industrially most important group of secondary metabolites. Since two thirds of the clinically useful antibiotics of natural origin are produced by *Streptomyces* (Kieser *et al.*, 2000) it is of major interest to understand how these filamentous soil bacteria synthesize antibiotics, to allow manipulation of biosynthetic pathways for production of novel compounds with improved properties. Further investigations focus on the identification and characterisation of genes involved in the regulation of antibiotic biosynthesis, and the use of the resulting knowledge to enhance secondary metabolite production.

Biosynthesis of secondary metabolites in Streptomycetes is a complex process involving different mechanisms of regulation. Two phylogenetically distant species have received especially extensive attention so far: *S. coelicolor* A3(2) because of its early development to a thoroughly studied genetic system which allowed the analysis of the interrelation between morphological differentiation and secondary metabolite formation; and *S. griseus* because it provided the first well-studied bacterial example of extracellular signalling by a hormone-like lactone, the γ-butyrolactone A-factor (Chater & Horinouchi, 2003). In both organisms, antibiotic production is affected by pleiotropic regulatory genes that influence the expression of pathway-specific regulatory genes which are clustered together with the structural genes of the biosynthetic enzymes.

In *S. griseus*, transcription of streptomycin biosynthetic genes is activated by StrR, a pathway-specific regulatory protein which binds to DNA loci containing an inverted repeat with the consensus sequence GTTCGActG(N)11CagTcGAAc. These loci are situated upstream of the respective promoter regions (Retzlaff & Distler, 1995; Tomono *et al.*, 2005). Four StrR binding sites have been identified in the streptomycin biosynthetic gene cluster in *S. griseus* and three in *S. glaucescens*. StrR contains a putative helix-turn-helix (HTH) motif (Retzlaff & Distler, 1995) which is typical for a family of bacterial and phage DNA-binding proteins (Pabo & Sauer, 1992).
ActII-ORF4 from *S. coelicolor* (Arias *et al.*, 1999; Wietzorrek & Bibb, 1997) and *Dnrl* from *S. peucetius* (Sheldon *et al.*, 2002; Wietzorrek & Bibb, 1997) belong to another family of regulatory proteins (SARPs). Their predicted secondary structure is an OmpR-like DNA-binding domain, and this structure is different from the typical HTH motif (Sheldon *et al.*, 2002; Wietzorrek & Bibb, 1997). These proteins act as transcriptional activators of target genes by binding to DNA loci that contain direct heptameric repeats with the consensus sequence TCGAGCG/C. These loci are situated close to the transcriptional start sites (Arias *et al.*, 1999; Wietzorrek & Bibb, 1997).

Another extensively studied system is the regulation of tylosin biosynthesis in *Streptomyces fradiae*. Tylosin biosynthesis is activated by two positive regulatory genes *tylS* and *tylR*, which act in a cascade-like reaction mechanism, and their overexpression could be used to enhance tylosin production (Bate *et al.*, 2006; Bibb, 2005).

In other *Streptomyces* strains further types of pathway-specific regulatory genes have been found, like *smrR* in *S. ambofaciens* which encoded protein shows no significant sequence similarity to any other known regulatory protein; the negative regulator *mmyR* of the methylenomycin cluster in *S. coelicolor*; and *dnrN* of the daunorubicin cluster in *S. peucetius* as well as *redZ* of the undecylprodigiosin cluster in *S. coelicolor*, both representing response regulatory genes of two-component systems. However, in some pathways no regulatory gene has been found, i.e. in erythromycin pathway (Rawlings, 2001).

Large-scale industrial fermentation requires strains producing high amounts of compound to ensure cost effective production, which has been traditionally obtained by strain improvements, i.e. expression of the biosynthetic gene cluster in a heterologous host or in an industrially optimized strain. Increased secondary metabolite production has additionally been obtained by i) metabolic engineering redirecting primary metabolic fluxes by introduction of genetic modifications through recombinant DNA technology, in a manner to support high secondary metabolite productivities (Adrio & Demain, 2006; Nielsen, 1998), ii) engineering antibiotic resistance, i.e. actinorhodin and undecylprodigiosin in *S. coelicolor* and *S. lividans* by introducing point mutations in the *rspL* gene, encoding the ribosomal protein S12,
I. INTRODUCTION

which confers resistance to streptomycin (Okamoto-Hosoya et al., 2003; Shima et al., 1996), iii) deregulation of the expression of secondary metabolite pathways, by overexpressing pathway-specific positive regulators, i.e. actinorhodin and undecylprodigiosin in *S. coelicolor* by actII-orf4 and redD (Fernández-Moreno et al., 1991; Narva & Feitelson, 1990), undecylprodigiosin in *S. lividans* and *S. parvulus* by redD (Malpartida et al., 1990), nikkomycin in *S. ansochromogenes* by sanG (Liu et al., 2005) and clavulanic acid in *S. clavuligerus* by ccaR (Hung et al., 2007; Pérez-Llarena et al., 1997) or vice versa, by inactivation of pathway repressors, i.e. chromomycin in *S. griseus* subsp. *griseus* by inactivation of the transcriptional repressor cmmRII (Menendez et al., 2007). Subsequently, the exchange of regulatory genes and promoter regions with ermEp*, representing a constitutive promoter, has been reported not only to abolish secondary metabolite production from its origin regulation circuit, but also to result in enhanced production titers, i.e. the replacement of four regulatory genes including the promoter region P_J in the jadomycin gene cluster by ermEp* resulted in increased jadomycin B production in *Streptomyces venezuelae* (Zheng et al., 2004).

I.3. Aminocoumarin antibiotics

The closely related aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A₁ are produced by different *Streptomyces* strains. Novobiocin is produced by *S. spheroides* NCIMB 11891 (Berger et al., 1978) and *S. niveus* (Smith et al., 1956). However, Lanoot et al. have proposed that these two strains are synonyms of *S. caeruleus* LMG 19399T (Lanoot et al., 2002). Clorobiocin is produced by *S. hygroscopicus*, *S. albocinerescens* and *S. roseochromogenes* var. *oscitans* (Mancy et al., 1974; Ninet et al., 1972). Coumermycin A₁ is produced by *S. rishiriensis*, *S. hazeliensis* var. *hazeliensis*, *S. spinichromogenes* and *S. spinicoumarensis* (Berger et al., 1978; Kawaguchi et al., 1965). Simocyclinone D8 (Schimana et al., 2000) and rubradirin (Sohng et al., 1997) are two further aminocoumarins found in nature, which are also produced by different *Streptomyces* strains.
I.3.1. Chemical structure

Novobiocin, Clorobiocin and Coumermycin A₁ contain three structural moieties: a 3-amino-4,7-dihydroxycoumarin moiety (named Ring B), a deoxysugar moiety (named Ring C) and an acyl component (Fig. I.1). Ring B is linked to the acyl component via an amide bond, and to Ring C via a glycoside bond. Clorobiocin and novobiocin share the same 3-dimethylallyl-4-hydroxybenzoyl moiety (named Ring A) as acyl component. Clorobiocin differs from novobiocin at two positions: novobiocin bears a carbamoyl group at position 3 of the noviose moiety, while clorobiocin contains a 5-methylpyrrole-2-carboxylic acid, and clorobiocin, as indicated by its name, carries a chlorine atom at position 8 of Ring B, while novobiocin carries a methyl group. Coumermycin A₁ contains two noviosyl aminocoumarin moieties, and carries a different acyl component in comparison to novobiocin and clorobiocin, i.e. a 3-methylpyrrole-2,4-dicarboxylic acid. Coumermycin A₁ contains, similar to novobiocin, a methyl group at position 8 of Ring B and contains, similar to clorobiocin, the same 5-methylpyrrole-2-carboxylic acid at position 3 of the noviose moiety (Fig. I.1).

Simocyclinone D8 and rubradirin contain the same aminocoumarin ring as the three closely related aminocoumarin antibiotics described above. Furthermore, simocyclinone D8 carries a chlorine atom at position 8 of its aminocoumarin ring just as clorobiocin. In contrast to novobiocin, clorobiocin and coumermycin A₁, simocyclinone D8 and rubradirin do not carry a noviose moiety, and their acyl components are large and complicated structures (Fig. I.1).
I.3.2. Mechanism of action and clinical application

Novobiocin, Clorobiocin and Coumermycin A₁ are potent inhibitors of bacterial DNA gyrase. The affinity of these drugs to bacterial gyrase is extremely high. The equilibrium dissociation constants (KDs) are in the 10 nM range, i.e. two orders of magnitude lower in comparison to modern fluoroquinolones. Gyrase belong to type II topoisomerase, which are present in all cell types. DNA topoisomerases are essential for cell viability and involved in important DNA-processes, i.e. DNA transcription, recombination and replication. The presence of DNA-gyrase in all bacteria, but not in eukaryotes makes it to a good target for antibacterial agents. With regard to its function DNA-Gyrase is responsible for the ATP-dependent introduction of negative supercoils into double-stranded DNA. It consists of two subunits: GyrA and GyrB, which together build up the active enzyme complex, an A₂B₂ heterodimer. DNA
gyrase has already been isolated from many different bacteria, but so far the one by \textit{Escherichia coli} represents the best characterised. GyrA, a protein of 97 kDa, includes an N-terminal domain involved in breakage-resealing reactions and a C-terminal domain, involved in the DNA-protein interaction. GyrB, a protein of 90 kDa, also includes a N-terminal and a C-terminal domain. While the N-terminus (43 kDa) catalyses ATP hydrolysis, the C-terminus interacts with GyrA and DNA (Maxwell, 1997; Maxwell, 1999).

All three aminocoumarin antibiotics compete with ATP for binding to the B subunit of DNA gyrase and inhibit the ATP-dependent DNA supercoiling catalysed by gyrase (Maxwell, 1999; Maxwell & Lawson, 2003). In comparison, fluorquinolones inhibit gyrase-catalysed supercoiling by disrupting the DNA breakage-reunion reaction on GyrA (Jacoby, 2005). X-ray crystallographic studies with the 24 kDa N-terminal subdomain of \textit{Escherichia coli} GyrB showed that both, the aminocoumarin and the substituted deoxysugar moieties of these substances are essential for their binding to the B-subunit of Gyrase. In these studies the resulting complexes between novobiocin (Lewis et al., 1996) and clorobiocin (Tsai et al., 1997) and the protein involve hydrophobic interactions and a network of hydrogen bonds. Especially hydrogen bonds between Arg136 and the aminocoumarin ring, Asp73 and the 3-acyl group on the deoxysugar, and Asn46 and the 2-hydroxyl group on the deoxysugar represent key bonds. The drugs do not occupy the same binding-pocket as ATP, but the sites for the two ligands overlap: the deoxysugar moiety overlaps the binding-site for the adenine ring of ATP.

Further studies showed that Coumermycin A₁ stabilize a dimer form of the 43 kDa N-terminal fragment of GyrB. This is consistent with the pseudo-dimeric structure of coumermycin (Fig. I.1)(Maxwell & Lawson, 2003).

Topoisomerase IV (topo IV), another bacterial type II topoisomerase has been proposed of being a further target of novobiocin (Hardy & Cozzarelli, 2003). Topoisomerase IV has a structure similar to bacterial DNA-gyrase: two ParC and two ParE subunits building up a C₂E₂-complex. ParC is responsible for DNA breakage and reunion, and ParE contains the ATP binding-site. Mutational experiments in the ParE subunit led to the conclusion, that novobiocin inhibits topo IV by the same mechanism as DNA gyrase is being inhibited.
Aminocoumarin antibiotics show a strong activity against gram-positive bacteria, including methicillin- and vancomycin-resistant *Staphylococcus* strains. Novobiocin (Albamycin®, Pharmacia & Upjohn) was licensed as drug in the USA for the treatment of human infections with multi-resistant gram-positive bacteria such as *Staphylococcus aureus* and *S. epidermidis*. Its efficacy has been demonstrated in preclinical and clinical trials (Raad *et al.*, 1995; Raad *et al.*, 1998; Walsh *et al.*, 1993). However, clinical use of these antibiotics remains restricted, due to some adverse reactions (principally urticaria and dermatitis), their poor solubility in water, and their low activity against gram-negative bacteria (resulting from poor permeability) (Maxwell, 1993). In comparison, clorobiocin and coumermycin have not been used clinically. Therefore, the generation of new, structurally modified aminocoumarin antibiotics, and of course the test whether they may be able to overcome limitations of the known compounds, is still of major interest (Maxwell & Lawson, 2003).

Further studies showed that aminocoumarins act synergistically with antitumour drugs (Lorico *et al.*, 1992; Rappa *et al.*, 1992; Rappa *et al.*, 2000a; Rappa *et al.*, 2000b). Additionally, interactions with eukaryotic heat shock protein 90 (Hsp90) have been described (Burlison & Blagg, 2006; Burlison *et al.*, 2006; Huang & Blagg, 2007).

### I.3.3. Structure-activity relationships

*In vitro* investigations on the inhibitory activity of aminocoumarin antibiotics and their analogues towards *E. coli* DNA gyrase as well as investigations on their antibacterial activity against *E. coli* cells showed, that the aminocoumarin moiety linked to the substituted deoxysugar is important and that lack of the pyrrole or carbamoyl substituent resulted in loss of inhibitory activity (Hooper *et al.*, 1982). Furthermore, novenamine, i.e. aminocoumarin moiety plus substituted deoxysugar has been described to represent the minimal structural entity of novobiocin to obtain interaction with DNA gyrase (Reusser & Dolak, 1986). This finding is in accordance with X-ray data presented above. The aminocoumarin and deoxysugar moiety alone did not show any activity in antibacterial and anti-gyrase activities (Althaus *et al.*, 1988; Hooper *et al.*, 1982; Reusser & Dolak, 1986).

Coumermycin A₁ was the most active compound (Hooper *et al.*, 1982). As mentioned above, it has been speculated that this effect may be caused by its pseudo-dimeric
structure (Maxwell & Lawson, 2003). In comparison to many aryl and alkyl
substituents tested, the prenylated benzoic acid moiety of novobiocin and clorobiocin,
i.e. Ring A, was the most effective (Galm et al., 2004; Hooper et al., 1982). Ring A
was supposed to be unimportant in DNA gyrase interactions and to only facilitate the
absorption of aminocoumarin compounds (Lewis et al., 1996), which, however, was
disproved (Freitag et al., 2004; Galm et al., 2004; Lafitte et al., 2002).

Clorobiocin has been reported to show a higher inhibition of Escherichia coli gyrase
and bacterial growth than novobiocin (Hooper et al., 1982), and to bind more
efficiently to isolated gyrase (Lafitte et al., 2002; Lewis et al., 1996; Tsai et al., 1997).
Most authors have attributed the higher activity of clorobiocin primarily to the pyrrole
moiety at C-3 of the deoxysugar moiety (Berger et al., 1978; Tsai et al., 1997).
Interestingly, however, clorobiocin acid, the aglycon of clorobiocin, but not novobiocin
acid, was found to inhibit both DNA synthesis in vivo and gyrase activity in vitro
(Althaus et al., 1988; Reusser & Dolak, 1986), suggesting that the chlorine atom
makes an important contribution to the biological activity of this molecule.

I.3.4. Biosynthetic gene clusters

Structural differences and similarities between novobiocin, clorobiocin and
coumermycin A1 have been found to be remarkably well reflected by differences and
similarities in the organization of the respective biosynthetic gene clusters (Pojer et
al., 2002) (Fig. I.2).

In addition to candidate genes for the biosynthetic reactions, in all three clusters a
gene for aminocoumarin-resistant gyrB subunit was found downstream of the
biosynthetic genes for the deoxysugar. The clorobiocin and coumermycin A1 clusters
contain a second aminocoumarin-resistant gyrB homologue, i.e. parY\textsuperscript{R} (Fig. I.2).

The genes located upstream of nov/clo/couE and downstream of the resistance
genes are completely different for each cluster (Eustáquio et al., 2003; Schmutz et al.,
2003). These genes may be involved in the primary metabolism, indicating that
nov/clo/couE and gyrB\textsuperscript{R}/parY\textsuperscript{R} may represent the left and the right borders of these
clusters respectively (Eustáquio et al., 2003; Schmutz et al., 2003).
I.3.4.1. Novobiocin biosynthesis

The novobiocin biosynthetic gene cluster spans 23.4 kb and comprises 20 coding sequences (Li & Heide, 2004; Li et al., 2006; Li & Heide, 2006). The genes novHIJKLM are responsible for the synthesis and the linkage of the aminocoumarin moiety. novQR are responsible for the generation of the prenylated 4-hydroxybenzoate moiety, and novSTUVW for the generation of the deoxysugar. novN, novO and novP encode enzymes involved in tailoring reactions, i.e. the carbamoylation and methylation of the novobiocin skeleton. novF is probably responsible for the availability of 4-hydroxyphenylpyruvate, a precursor of both aromatic moieties of novobiocin.
I.3.5. Resistance genes

Biosynthesis of antibiotics with high biological activity requires mechanisms to protect the producer against the inhibitory effect of these compounds. It has been shown that the resistance mechanism of the novobiocin producer *S. spheroides* is based on the *de novo* synthesis of the aminocoumarin-resistant GyrB subunit (Thiara & Cundliffe, 1988). This is obtained by the replacement of the aminocoumarin-sensitive GyrB^S^ subunit in the active heterodimer of bacterial DNA-gyrase by GyrB^R^-protein. Therefore, the respective *gyrBR* gene was identified in all three clusters, as expected, since the producers must obviously protect their gyrases from the inhibitory effect of aminocoumarins during antibiotic production. In addition to *gyrBR*, the coumermycin A_1_ and clorobiocin, but not the novobiocin cluster contain a further gene, i.e. *parYR*, with high sequence similarity to the B-subunit of type II topoisomerases. Heterologous expression of *gyrBR* and *parYR* in *S. lividans* TK24 resulted in similar levels of resistance against novobiocin and coumermycin A_1_ (Schmutz *et al.*, 2003).
Furthermore, it has been shown that GyrB\textsuperscript{R} leads to resistance against novobiocin and coumermycin A\textsubscript{1}. Heterologous expression and purification of GyrB\textsuperscript{R} and ParY\textsuperscript{R} of the coumermycin A\textsubscript{1} gene cluster and the corresponding topoisomerase subunits GyrA and ParX of \textit{Streptomyces coelicolor} showed, that ATP-dependent DNA supercoiling is catalysed by the \textit{in vitro} complex of GyrA and GyrB\textsuperscript{R}. For the complex of ParX and ParY\textsuperscript{R} an ATP-dependent function of decatenation and relaxation of DNA was demonstrated. This function was usually assigned to the topoisomerase IV (Schmutz \textit{et al.}, 2004). This finding strongly supports the hypothesis, that topo IV may represent a further target for aminocoumarins.

I.3.6. Regulation of aminocoumarin-antibiotic production

Cloning and sequencing of the biosynthetic gene clusters of the aminocoumarin antibiotics (Pojer \textit{et al.}, 2002; Steffensky \textit{et al.}, 2000; Wang \textit{et al.}, 2000) allowed detailed investigations of the biosynthetic pathways (Li & Heide, 2004) as well as the generation of novel antibiotics by metabolic engineering, chemo-enzymatic synthesis and precursor-directed biosynthesis (Li & Heide, 2005).

On the other hand, only limited knowledge is available on how aminocoumarin antibiotic production is regulated. The novobiocin biosynthetic gene cluster contains two putative regulatory genes, \textit{novE} and \textit{novG}. Close orthologues of both genes are found in the gene clusters of clorobiocin and coumermycin A\textsubscript{1}.

NovG shows 41 % identity at the amino acid level to StrR, the pathway-specific transcriptional activator of streptomycin biosynthesis in \textit{Streptomyces glaucescens} and \textit{S. griseus} (Retzlaff & Distler, 1995; Thamm & Distler, 1997; Tomono \textit{et al.}, 2005). The predicted amino acid sequence of NovG shows a putative HTH motif in the central region of the protein, which is typical for a family of bacterial and phage DNA-binding proteins (Pabo & Sauer, 1992). This motif is also found in StrR (Retzlaff & Distler, 1995; Thamm & Distler, 1997).

Since the natural novobiocin producer \textit{Streptomyces spheroides} is difficult to manipulate genetically (Hussain & Ritchie, 1991), the biosynthetic gene cluster of novobiocin was expressed in \textit{S. coelicolor} M512 (Eustáquio \textit{et al.}, 2005a) which genome sequence (of the wild-type strain \textit{S.coelicolor}A3(2)) is available (Bentley \textit{et al.}, 2002).
In comparison to the natural producer strain the heterologous host showed equal novobiocin production titers (Eustáquio et al., 2005a). However, engineering of the novobiocin pathway is much easier in the heterologous host than in the wild-type strain.

Inactivation of \textit{novG} led to a 98\% reduction of the novobiocin productivity of the heterologous producer strain (Eustáquio et al., 2005b). Due to the fact that \textit{ΔnovG} strains still produced some novobiocin, an essential catalytic role for \textit{novG} could be ruled out. This finding also indicated a low level expression of the biosynthetic genes in absence of NovG. Introduction of \textit{novG} with its own putative promoter (i.e. 336 bp upstream of the start codon of \textit{novG}) in the expression vector pWHM3 into the \textit{ΔnovG} strain led to restoration of novobiocin production nearly to the same level (80\%) as observed before \textit{novG} inactivation, indicating that the observed phenotype of \textit{ΔnovG} strains was indeed caused by the lack of \textit{novG} (Eustáquio et al., 2005b).

In comparison, expression of \textit{novG} from a multicopy plasmid in a \textit{S. coelicolor} M512 strain carrying the intact novobiocin cluster resulted in a 2.7-fold overproduction of novobiocin, suggesting that novobiocin biosynthesis in the heterologous expression host is limited by availability of the activator protein (Eustáquio et al., 2005b).

As mentioned above, NovG shows sequence similarity to StrR a positive regulator of streptomycin biosynthesis. StrR binds to DNA loci containing an inverted repeat with the consensus sequence GTTCGActG(N)11CagTcGAAc. These loci are situated upstream of the respective promoter regions (Retzlaff & Distler, 1995).

EMSA (electrophoretic mobility shift assays), in which selected DNA fragments of the novobiocin biosynthetic gene cluster were used, demonstrated that His\textsubscript{6}-tagged NovG protein binds specifically to the \textit{novG-novH} intergenic region (Eustáquio et al., 2005b). Therefore, it has been speculated that this binding may activate transcription of \textit{novH}, just as described for StrR, which activates transcription of streptomycin biosynthetic genes by binding to their promoter regions (Retzlaff & Distler, 1995), (Tomono et al., 2005). The putative binding-site of NovG is located directly downstream of the translational stop codon of \textit{novG}, i.e. between -165 and -194 bp upstream of the putative translational start codon of \textit{novH}. Due to the fact that EMSA was carried out with nickel affinity purified protein, generated in \textit{E. coli}, it is very likely that NovG binds to DNA with no further macromolecular factor involved. However,
the possibility that other proteins may be required for activating the transcription of novH may not completely be ruled out.

As mentioned above, the novobiocin and clorobiocin gene clusters are highly similar to each other on amino acid level. It was demonstrated that NovG could also bind to a DNA region of the clorobiocin cluster, i.e. the cloG-cloY intergenic region; the putative binding-site is located between positions -160 and -189 upstream of the putative translational start codon of cloY (Eustáquio et al., 2005b).

The in silico analysis of the DNA fragments from the novobiocin and clorobiocin clusters which bind NovG showed the presence of a conserved 9 bp inverted repeat, separated by a less-conserved (two mismatches) 11 bp spacer sequence (Eustáquio et al., 2005b). The previously identified StrR binding sites in S. griseus and S. glaucescens contain a similar palindromic structure, i.e. conserved inverted repeats of 9 bp each, separated by a non-conserved 11 bp spacer (Retzlaff & Distler, 1995). The same putative NovG binding-site, with exactly the same inverted repeat and spacer sequences as found upstream of cloY, is also present in the coumermycin A₁ cluster, between genes couG and couY. The close similarity of the putative NovG/CloG/CouG binding-sites in the novobiocin, clorobiocin and coumermycin A₁ clusters further indicates a common evolutionary origin for these clusters (Eustáquio et al., 2003).

The consensus sequence GTTCRACTG(N)₁₁CRGTYYGAAC or similar motifs were not found anywhere else in the gene clusters of novobiocin and clorobiocin, except in the mentioned regions upstream of novH and cloY, respectively. In contrast, four StrR binding sites have been identified in the streptomycin biosynthetic gene cluster in S. griseus and three in the 5´-OH-streptomycin cluster in S. glaucescens (Retzlaff & Distler, 1995).

The predicted gene product of novE comprises 217 amino acids and contains the rare TTA-leucine codon (as codon 189) which suggests its dependence on bldA, the structural gene for tRNA^{UUA}. This may indicate a regulatory role of NovE in novobiocin biosynthesis since most of the known TTA-containing genes specify regulatory or resistance proteins associated with biosynthetic gene clusters for antibiotics (Leskiw et al., 1991). In addition to that, NovE shows 45 % identity at the
amino acid level to LmbU in the lincomycin biosynthetic gene cluster. It has been speculated that lmbU may code for a regulatory protein (Peschke et al., 1995). Orthologues of novE and lmbU include cloE and couE from the clorobiocin and coumermycin gene clusters, rubC4 from the gene cluster of the aminocoumarin antibiotic rubradirin (GenBank AJ871581) and five further genes deposited in the database. All of these genes have been found in actinobacteria. However, their function is yet unknown.

Heterologous expression experiments showed that the expression of the complete novobiocin cluster, i.e. nov-BG1, including several kb of DNA upstream of the start codon of novE (Fig. I.4), in the heterologous host Streptomyces coelicolor M512 resulted in a high production of novobiocin. In contrast, the expression of a similar construct, i.e. novAE4, including only 180 bp upstream of the novE start codon (Fig. I.4) had given only 5 % of the production achieved with nov-BG1 (Eustáquio et al., 2005a). Subsequently, the expression of a construct which contained 484 bp upstream of the novE start codon (Fig. I.4), i.e. nov-AE12, resulted in 12 times higher production levels in comparison with nov-AE4 (Dangel et al., 2008). The additional DNA region contained in nov-AE12 but not in nov-AE4 comprises only the 3′-end of the structural gene novD, without a start codon. Therefore, this effect cannot be attributed to the expression of a functional NovD protein. This finding may represent an indication that the promoter region of novE extends more than 180 bp upstream of the novE start codon, and that expression of novE is important for novobiocin biosynthesis.

Fig. I.4: Schematic comparison of the inserts of cosmids nov-BG1, nov-AE12 and nov-AE4.
In the course of investigations on the genes at the borders of the novobiocin, clorobiocin and coumermycin gene clusters, a replacement of novE with an apramycin resistance gene has been carried out in the novobiocin producer *Streptomyces spheroides* (syn. *Streptomyces caeruleus*) (Eustáquio et al., 2003). This resulted in a reduction of the novobiocin production by 96 % but not in a complete abolishment. This result indicated that novE may act as a positive regulator of novobiocin biosynthesis. However, downstream effects of the introduced apramycin resistance cassette can not be excluded.
I. INTRODUCTION

I.4. Aims of this study

The first aim of this study was to supply functional evidence for the regulatory role of novE in novobiocin biosynthesis. For this reason the following experiments have been carried out:

- Inactivation of novE in cosmid nov-BG1 and heterologous expression of the resulting ΔnovE-cosmid in Streptomyces coelicolor M512.
- Complementation of the ΔnovE-mutation by introducing an intact copy of novE on a multicopy number plasmid (pWHM3-based) into Streptomyces coelicolor M512 (ΔnovE).
- Overexpression of an intact copy of novE on a multicopy number plasmid (pWHM3-based) in Streptomyces coelicolor M512 (nov-BG1).
- Introduction of the positive regulator novG under control of the constitutive ermE* promoter (pUWL-based) into Streptomyces coelicolor M512 (ΔnovE).
- HPLC analysis of secondary metabolite production in the generated mutants.

The second aim of this study was to show whether novE and novG act in a cascade-like or a parallel mechanism. Therefore, the following experiments have been carried out:

- Introduction of an intact copy of novG under control of its own putative promoter on a multicopy number plasmid (pWHM3-based) into Streptomyces coelicolor M512 (ΔnovE).
- Introduction of an intact copy of novE under control of its own putative promoter on a multicopy number plasmid (pWHM3-based) into Streptomyces coelicolor M512 (ΔnovG).
- Establishment of methods for RNA isolation from Streptomyces and for RT-PCR.
- RT-PCR analysis for investigations on novG transcription in Streptomyces coelicolor M512 (ΔnovE) and novE transcription in Streptomyces coelicolor M512 (ΔnovG).

Further aims were the determination of promoter regions and investigations on the transcriptional regulation of the novobiocin biosynthetic gene cluster. Investigations on the determination of promoter regions within the novobiocin biosynthetic gene
cluster were carried out in cooperation with Johannes Härle as diploma student and included the following experiments:

- Introduction of Ω (omega) interposons downstream of putative promoter regions into cosmid nov-BG1, resulting in interruption of transcription up to the next active promoter region.

- Heterologous expression of the modified constructs in *Streptomyces coelicolor* M512.

- RT-PCR analysis of the generated mutants.

Investigations on the transcriptional regulation required the establishment of quantitative RT-PCR. Quantitative RT-PCR experiments were carried out for contribution of the promoter regions within the region from *novH* to *novW* and for investigations on the interplay of *novE* and *novG* as well as on the influence of *novE*- and *novG*-inactivation on the transcription of novobiocin biosynthetic genes and the resistance gene *gyrB*.

Subsequently, the final aim of this study was the generation of *Streptomyces coelicolor* M512 strains with improved novobiocin production, independent from its origin regulation cascade. Therefore the following experiments were carried out:

- Generation of *S. coelicolor* M512 (Δ*novE*Δ*novG*).

- Introduction of tetracycline-controllable-promoters (tcp830) into cosmid nov-BG1 in exchange for the entire *novEFG*-region and/or for the intergenic region between *novP* and *novQ*.

- Heterologous expression of the modified cosmids in *Streptomyces coelicolor* M512.

- HPLC analysis of secondary metabolite production, with and without induction by anhydrotetracycline.
II. MATERIALS AND METHODS

II.1. Chemicals

Chemicals and components of the media used in this thesis are listed in Table II.1.

TABLE II.1: Chemicals and media components

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Chemical / Media component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amersham Biosciences, Freiburg, Germany</td>
<td>Agarose</td>
</tr>
<tr>
<td>Bacto-Difco, Heidelberg, Germany</td>
<td>Agar, Casaminoacids, Malt extract, Peptone, Tryptic soy broth, Tryptone, Yeast extract</td>
</tr>
<tr>
<td>Calbiochem-Novabiochem, Bad Soden, Germany</td>
<td>L-Proline, Thiostrepton</td>
</tr>
<tr>
<td>Fluka, Ulm, Germany</td>
<td>Apramycin, Novobiocin</td>
</tr>
<tr>
<td>FMC BioProducts, Rockland, USA</td>
<td>NuSieve® GTG® Agarose</td>
</tr>
<tr>
<td>Merck, Darmstadt, Germany</td>
<td>Chloramphenicol, EDTA, Ethanol, Glucose, Meat extract, Methanol</td>
</tr>
<tr>
<td>Roth, Karlsruhe, Germany</td>
<td>Anhydro-tetracycline (aTc), 5-Bromo-4-chlor-3-indolyl-β-D-galactopyranoside (X-Gal), Carbenicillin, Glacial acetic acid, Glass beads, Glycine, Isopropanol, Isopropyl-β-thiogalactoside (IPTG), Maleic acid, Na-salicylat, Phenol/Chloroform/Isoamylalkohol(25:24:1), Phenol (pH 8.0), Polyethylene glycol (PEG) 1000, Sodium dodecyl sulphate (SDS)</td>
</tr>
</tbody>
</table>
II. MATERIALS AND METHODS

II.2. Materials for Chromatography

The liquid chromatography media were obtained as commercial columns. The storage of the columns was carried out according to the manufacturers' instructions.

TABLE II.2: Liquid chromatography media

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+S Chromatographie Service, Düren, Germany</td>
<td>Multosphere® RP 18-5 (commercial column, 5 µm, 150×4,6 mm)</td>
</tr>
</tbody>
</table>

II.3. Enzymes and kits

TABLE II.3: Enzymes and kits

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Enzymes and kits</th>
</tr>
</thead>
</table>
| Amersham Biosciences, Freiburg, Germany | Restriction endonucleases  
T4 DNA Ligase |
| Fermentas | Deoxyribonuclease I (1 U/µl)  
10 x buffer with MgCl₂ (100 mM Tris-HCl (pH 7.5 at 25 °C), 25 mM MgCl₂, 1 mM CaCl₂)  
Random hexamer primers (0.2 µg/ml)  
Revert-Aid™M-MuLV Reverse Transcriptase (200 U/µl)  
5 x reaction buffer (250 mM Tris-HCl (pH: 8.3 at 25 °C), 250 mM KCl, 20 mM MgCl₂, 50 mM DTT)  
RiboLock™ Ribonuclease Inhibitor (20 U/µl) |
| Sigma-Aldrich, Deisenhofen, Germany | N-Lauroylsarcosine (Na-Salt, 35%)  
Bromophenol blue  
Dimethyl formamide (DMF)  
Dimethyl sulfoxide (DMSO)  
Ethylene glycol  
Kanamycin  
Polyoxyethylenesorbitan monolaurate (Tween 20)  
Tetracycline  
Tris base |
| Südzucker, Mannheim, Germany | Sucrose |
## II. MATERIALS AND METHODS

### II.4. Media, buffers and solutions

#### II.4.1. Media for bacterial cultivation

The media used in this study were as follows. Unless otherwise stated, the media were prepared with distilled water and autoclaved for 20 min at 121 °C (15 psi). To obtain solid media, 2% (w/v) agar was added before autoclaving. If necessary, sterile supplementary components like antibiotics and other heat-labile substances were added in the sterile media at time of use. The media were stored at room temperature or at 4 °C.

#### II.4.1.1. Cultivation of *E. coli*

**LB (Luria-Bertani) Medium** (Sambrook & Russell, 2001)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0 g</td>
</tr>
</tbody>
</table>
Dissolve the ingredients in about 900 ml water, adjust the pH to 7.0, and adjust the volume to 1 litre with water. Sterilize by autoclaving.

**SOB Medium**

- Tryptone 20.0 g
- Yeast extract 5.0 g
- NaCl 0.5 g

Dissolve the ingredients in about 900 ml water, adjust the pH to 7.0, and adjust the volume to 1 litre with water. Sterilize by autoclaving. Add 10 ml of a sterile solution of 1 M MgCl₂.

**II.4.1.2. Cultivation of Streptomyces**

**YMG (Yeast-Malt-Glucose) Medium**

- Yeast extract 4.0 g
- Malt extract 10.0 g
- Glucose 4.0 g

Dissolve the ingredients in about 900 ml water, adjust the pH to 7.3, and add water to make up to 1 litre. Sterilize by autoclaving.

**TSB (Tryptone Soya Broth) Medium (Kieser et al., 2000)**

- Tryptone Soya Broth 30.0 g

Dissolve the ingredient in up to 1 litre water, and sterilize by autoclaving.

**MS (Mannitol Soya flour) Agar (Kieser et al., 2000)**

- Mannitol 20.0 g
- Soya flour 20.0 g
- Agar 20.0 g

Dissolve the mannitol in up to 1 litre tap water and pour 100 ml into 300 ml Erlenmeyer flasks each containing 2 g agar and 2 g soya flour. Sterilize by
II. MATERIALS AND METHODS

autoclaving twice (115 °C, 15 min) with gentle shaking between the two runs.

DNA (Difco Nutrient Agar) (Kieser et al., 2000)

Difco Nutrient Agar 4.6 g

Place the ingredient into 300 ml Erlenmeyer flasks and add 200 ml water. Sterilize by autoclaving.

MM Medium (Kieser et al., 2000)

L-asparagine 0.5 g
K₂HPO₄ 0.5 g
MgSO₄ · 7H₂O 0.2 g
FeSO₄ · 7H₂O 0.01 g
Mannitol 5 g
Agar 10 g
Distilled water ad 1000ml

Dissolve the ingredients, except agar, in the distilled water, adjust to pH 7.0-7.2 and dispense 200 ml into 300 ml Erlenmeyer flasks each containing 2 g agar. Close the flasks and sterilize by autoclaving.

II.4.1.3. Novobiocin production medium

CDM medium (Kominek, 1972)

tri-sodium citrate · 2H₂O 6.0 g
L-proline 6.0 g
K₂HPO₄ · 3H₂O 2.0 g
(NH₄)₂SO₄ 1.5 g
NaCl 5.0 g
MgSO₄ · 7H₂O 2.05 g
CaCl₂ · 2H₂O 0.4 g
ZnSO₄ · 7H₂O 0.1 g
Glucose (30% (w/v)) 100 ml
Dissolve the ingredients till NaCl in water, adjust the pH to 7.2, dissolve the remaining ingredients except the glucose solution, and add water to make up to 900 ml. Sterilize by autoclaving. Add the sterile glucose solution.

II.4.1.4. Protoplast transformation of *Streptomyces*

**Trace elements solution**

- ZnCl$_2$ 40 mg
- FeCl$_3$ · 6H$_2$O 200 mg
- CuCl$_2$ · 2H$_2$O 10 mg
- MnCl$_2$ · 4H$_2$O 10 mg
- Na$_2$B$_4$O$_6$ · 10H$_2$O 10 mg
- (NH$_4$)$_6$Mo$_7$O$_{24}$ · 4H$_2$O 10 mg

Dissolve in 1 litre distilled water and autoclave.

**R5 Medium** (Kieser *et al.*, 2000)

- Sucrose 103.0 g
- K$_2$SO$_4$ 0.25 g
- MgCl$_2$ · 6H$_2$O 10.12 g
- Glucose 10.0 g
- Difco Casaminoacids 0.1 g
- Trace elements solution 2.0 ml
- Difco Yeast extract 5.0 g
- TES buffer 5.73 g
- Agar (plates) 23.0 g

Dissolve in water to a final volume of 1 litre and sterilize by autoclaving. To prepare soft agar, 6 g Agar instead of 23 g, were added.

After autoclaving, add the following sterile solutions:

- KH$_2$PO$_4$ (0.5%) 10 ml
- CaCl$_2$ · 2H$_2$O (5 M) 4 ml
- L-Proline (20% (w/v)) 15 ml
- NaOH (1M) 7 ml
II. MATERIALS AND METHODS

II.4.2. Antibiotic solutions

Antibiotics were dissolved in appropriate solvents as stock solutions and kept at 
-20 °C. The aqueous solutions were sterilized by passing through a 0.22 µm filter. 
The solutions in ethanol and DMSO were autostereile. For antibiotic selection, the 
required antibiotics were added to the cooled media (room temperature to 60 °C) in 
appropriate concentration.

**TABLE II.4: Solutions of antibiotics**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration in stock solution (mg/ml)</th>
<th>Concentration in media (µg/ml)</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apramycin</td>
<td>50</td>
<td>15-50(^{a})</td>
<td>H(_2)O</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>50-100</td>
<td>50-100</td>
<td>H(_2)O</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>25-50</td>
<td>25-50</td>
<td>ethanol</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50</td>
<td>15-50(^{a})</td>
<td>H(_2)O</td>
</tr>
<tr>
<td>Anhydrotetracycline</td>
<td>50</td>
<td>1(^{b})</td>
<td>H(_2)O</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>12</td>
<td>12</td>
<td>ethanol</td>
</tr>
<tr>
<td>Thiostrepton</td>
<td>50</td>
<td>15-50(^{a})</td>
<td>DMSO</td>
</tr>
</tbody>
</table>

\(^{a}\)15 µg/ml in liquid and 50 µg/ml in solid media for selection of *Streptomyces*
strains; otherwise, 50 µg/ml.

\(^{b}\)anhydrotetracycline was used for induction of tcp830.

II.4.3. Buffers and solutions

Unless otherwise stated, the buffers and solutions were prepared with distilled water, 
autoclaved and stored at room temperature.

II.4.3.1. Buffers and Solutions for DNA isolation

**TABLE II.5: Buffers and solutions for plasmid and cosmid isolation from *E.coli***

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
<th>Final concentration</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution MP1</td>
<td>Tris-HCl</td>
<td>50 mM</td>
<td>Adjust the pH to 8.0. Add RNase A just before use.</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>10 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RNase A</td>
<td>100 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Solution MP2</td>
<td>NaOH</td>
<td>0.2 M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SDS</td>
<td>1% (w/v)</td>
<td></td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Solution</th>
<th>Components</th>
<th>Final concentration</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP3</td>
<td>KAc · 3H₂O</td>
<td>3 M</td>
<td>Adjust the pH to 4.8. Store at 4 °C.</td>
</tr>
</tbody>
</table>

**TABLE II.6: Buffers and solutions for plasmid isolation from *Streptomyces***

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
<th>Final concentration</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MP1GL</td>
<td>Glucose, Tris-HCl, EDTA, RNase A, Lysozyme</td>
<td>50 mM, 25 mM, 10 mM, 100 µg/ml, 2-4 mg/ml</td>
<td>Adjust the pH to 8.0. Add RNase A and lysozyme just before use.</td>
</tr>
<tr>
<td>MP2</td>
<td>NaOH, SDS</td>
<td>0.2 M, 1% (w/v)</td>
<td></td>
</tr>
<tr>
<td>MP3</td>
<td>KAc · 3H₂O</td>
<td>5 M</td>
<td>Adjust the pH to 4.8. Store at 4 °C.</td>
</tr>
</tbody>
</table>

**TABLE II.7: Buffers for isolation of genomic DNA from *Streptomyces***

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
<th>Final concentration</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSE buffer</td>
<td>Sucrose, Tris-HCl, EDTA, RNase A, Lysozyme</td>
<td>10.3%, 25 mM, 25 mM, 100 µg/ml, 3 mg/ml</td>
<td>Adjust the pH to 8.0. Add RNase A and lysozyme just before use.</td>
</tr>
<tr>
<td>2× Kirby mix</td>
<td>SDS, Sodium 4-aminosalicylate, 2 M Tris-HCl, pH 8, equilibrated phenol pH 8.0</td>
<td>2 g, 12 g, 5 ml, 6 ml</td>
<td>Dissolve the SDS and the sodium 4-aminosalicylate in up to 89 ml distilled water, add the Tris-HCl buffer and the phenol. Do not autoclave. Store protected from light at 4 °C.</td>
</tr>
<tr>
<td>TE buffer</td>
<td>Tris-HCl, EDTA</td>
<td>10 mM, 1 mM</td>
<td>Adjust the pH to 7.5.</td>
</tr>
</tbody>
</table>
II. MATERIALS AND METHODS

II.4.3.2. Buffers for DNA gel electrophoresis

TABLE II.8: Buffers for DNA gel electrophoresis

<table>
<thead>
<tr>
<th>Buffer/solution</th>
<th>Components</th>
<th>Final concentration</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>50×TAE</td>
<td>Tris base</td>
<td>2 M</td>
<td>Adjust the pH to 8.0 with glacial acetic acid.</td>
</tr>
<tr>
<td></td>
<td>EDTA</td>
<td>0.05 M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glacial acetic acid</td>
<td>57.1 ml/l</td>
<td></td>
</tr>
<tr>
<td>Load buffer</td>
<td>Glycerol</td>
<td>30% (w/v)</td>
<td>Store at 4 °C</td>
</tr>
<tr>
<td></td>
<td>Bromophenol blue</td>
<td>0.25% (w/v)</td>
<td></td>
</tr>
<tr>
<td>Ethidium bromide solution for staining the agarose gel</td>
<td>Ethidium bromide</td>
<td>1 µg/ml</td>
<td></td>
</tr>
</tbody>
</table>

II.4.3.3. Buffers and solutions for Southern blot analysis

TABLE II.9: Buffers and solutions for Southern blot analysis

<table>
<thead>
<tr>
<th>Buffer/solution</th>
<th>Components</th>
<th>Final concentration</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturing solution</td>
<td>NaOH</td>
<td>0.5 M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>1.5 M</td>
<td></td>
</tr>
<tr>
<td>Neutralizing buffer</td>
<td>Tris-HCl</td>
<td>0.5 M</td>
<td>Adjust the pH to 7.5.</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>3 M</td>
<td></td>
</tr>
<tr>
<td>20×SSC buffer</td>
<td>Trisodium citrate</td>
<td>0.3 M</td>
<td>Adjust the pH to 7.0.</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>3 M</td>
<td></td>
</tr>
<tr>
<td>Pre-hybridizing solution</td>
<td>Creamed milk powder</td>
<td>3%</td>
<td>Add to 5×SSC buffer before use.</td>
</tr>
<tr>
<td></td>
<td>SDS (10% (w/v) in H2O)</td>
<td>0.02%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N-lauroylsarcosine (35% (w/v) in H2O)</td>
<td>0.1%</td>
<td></td>
</tr>
<tr>
<td>Hybridizing solution</td>
<td>Creamed milk powder</td>
<td>1.5%</td>
<td>Add to 5×SSC buffer before use.</td>
</tr>
<tr>
<td></td>
<td>SDS (10% (w/v) in H2O)</td>
<td>0.02%</td>
<td>Add appropriate probe (5-25 ng/ml)</td>
</tr>
<tr>
<td></td>
<td>N-lauroylsarcosine (35% (w/v) in H2O)</td>
<td>0.1%</td>
<td></td>
</tr>
<tr>
<td>2×Washing buffer</td>
<td>SDS (10% (w/v) in H2O)</td>
<td>0.1%</td>
<td>Add to 2×SSC buffer before use.</td>
</tr>
<tr>
<td>0.5×Washing buffer</td>
<td>SDS (10% (w/v) in H2O)</td>
<td>0.1%</td>
<td>Add to 0.5×SSC buffer before use.</td>
</tr>
<tr>
<td>Maleic acid buffer</td>
<td>Maleic acid</td>
<td>0.1 M</td>
<td>Adjust the pH to 7.5.</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>0.15 M</td>
<td></td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>Tween® wash buffer</th>
<th>Tween® 20</th>
<th>0.3%</th>
<th>Add to the maleic acid buffer before use.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking solution</td>
<td>Creamed milk powder</td>
<td>3%</td>
<td>Add to the maleic acid buffer just before use</td>
</tr>
<tr>
<td>Detection buffer</td>
<td>Tris-HCl</td>
<td>0.1 M</td>
<td>Adjust the pH to 9.5</td>
</tr>
<tr>
<td>Antidote solution</td>
<td>NaCl</td>
<td>0.1 M</td>
<td></td>
</tr>
<tr>
<td>Stripping solution for blot</td>
<td>NaOH</td>
<td>0.2 M</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SDS (10% (w/v) in H₂O)</td>
<td>0.1%</td>
<td></td>
</tr>
</tbody>
</table>

II.4.3.4. Solutions for blue/white selection of *E. coli*

The storage was carried out at -20°C.

### TABLE II.10: Stock solutions for blue/white selection.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
<th>Per plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPTG</td>
<td>80 mg/ml in distilled water, sterilize by filtering</td>
<td>15 µl</td>
</tr>
<tr>
<td>X-Gal</td>
<td>20 mg/ml in DMF, autostereile</td>
<td>60 µl</td>
</tr>
</tbody>
</table>

II.4.3.5. Buffers for preparation of protoplasts and transformation of *Streptomyces*

The following sterile solutions were prepared separately. To obtain P-buffer and T-buffer, they were mixed according to the description and stored at -20°C.

### TABLE II.11: Buffers for preparation of protoplasts and transformation of *Streptomyces*

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
<th>Amount (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(protoplast)-buffer (Kieser <em>et al.</em>, 2000)</td>
<td>Sucrose (12% (w/v) in H₂O)</td>
<td>85.5</td>
</tr>
<tr>
<td></td>
<td>MgCl₂ 6H₂O (1M)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>K₂SO₄ (140 mM)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Trace elements solution</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>KH₂PO₄ (40 mM)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>CaCl₂ 2H₂O (250 mM)</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>TES (0.25M, pH 7.2)</td>
<td>10.0</td>
</tr>
</tbody>
</table>
II. MATERIALS AND METHODS

T(transformation)-buffer (Kieser et al., 2000)

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concentration</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose (25% (w/v) in H₂O)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Trace elements solution</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>K₂SO₄ (140 mM)</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>KH₂PO₄ (40 mM)</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>MgCl₂ · 6H₂O (1 M)</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>CaCl₂ · 2H₂O (5 M)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Tris-maleate (0.5 M, pH 8.0)</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>PEG 1000 (50% (w/v) in H₂O);</td>
<td>5.0</td>
<td>adjust the volume with distilled water to 10 ml.</td>
</tr>
</tbody>
</table>

II.4.3.6. Solution for isolation of RNA from *Streptomyces*

TABLE II.12: Solution for isolation of RNA from *Streptomyces*

<table>
<thead>
<tr>
<th>Solution</th>
<th>Components</th>
<th>Final concentration</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Kirby mix</td>
<td>N-lauroylsarcosine</td>
<td>1 % (w/v)</td>
<td>Dissolve N-lauroylsarcosine, sodium-salicylate in distilled water, add the Tris-HCl buffer and the phenol and add distilled water up to 100 ml. Do not autoclave. Store protected from light at 4°C.</td>
</tr>
<tr>
<td></td>
<td>Sodium-salicylate</td>
<td>6 % (w/v)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 M Tris-HCl pH 8.0</td>
<td>20 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Phenol pH 8.0</td>
<td>6 % (v/v)</td>
<td></td>
</tr>
</tbody>
</table>

II.5. Plasmids, bacterial strains, primers and probes

II.5.1. Vectors, cosmids and plasmids

TABLE II.13: Vectors, cosmids and plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>pGEM-T Linearized vector with T-overhang for direct cloning of PCR fragments with A-overhang, lacZα, ori, f1-origin, AmpR</td>
<td>Promega</td>
</tr>
</tbody>
</table>
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**Cosmid**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>nov-BG1</td>
<td>From cosmid 10-9C, <em>bla</em> gene replaced by cassette from pIJ787 (<em>oriT</em>, <em>tet</em>, <em>attP</em>, <em>int</em> φC31), KanR.</td>
<td>Bertolt Gust (Eustáquio et al., 2004)</td>
</tr>
<tr>
<td>nov-AE10</td>
<td>nov-BG1 (ΔnovG), KanR</td>
<td>(Eustáquio et al., 2005b)</td>
</tr>
<tr>
<td>nov-JH4</td>
<td>nov-BG1 (ΩnovH); KanR ApraR</td>
<td>Johannes Härle`s Thesis</td>
</tr>
</tbody>
</table>

**Plasmid**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIJ787</td>
<td>SuperCos1-derivative, <em>bla</em> gene replaced by a cassette containing <em>oriT</em>, <em>tet</em>, <em>attP</em>, <em>int</em> φC31, KanR.</td>
<td>Bertolt Gust (Eustáquio et al., 2004)</td>
</tr>
<tr>
<td>pUG019</td>
<td>pBlueskript SK(-)-derivative containing an apramycin resistance (<em>aac(3)IV</em>) cassette flanked by XbaI and SpeI restriction sites, AmpR</td>
<td>Ute Galm (Eustáquio et al., 2004)</td>
</tr>
<tr>
<td>pMS80</td>
<td>Plasmid containing the tetracycline-controllable promoter 830 (tcp830); ApraR</td>
<td>(Rodriguez-Garcia et al., 2005)</td>
</tr>
<tr>
<td>pAE8</td>
<td>1.43 kb <em>BamH</em>l-<em>EcoR</em>l fragment of pMS33 (novG, position 6 393 – 7 821, AF 170880) in the same sites of pWHM3, containing 336 bp before the putative start codon of novG; AmpR, ThioR.</td>
<td>(Eustáquio et al., 2005b)</td>
</tr>
<tr>
<td>pAE12</td>
<td>1.35 kb <em>PstI</em>-<em>Sph</em>l fragment of pAE11, cloned into the same sites of pWHM3 (same orientation as lacZ), containing novG and 336 bp upstream of its start codon (position 6383 - 7687 in AF170880); AmpR, ThioR.</td>
<td>Alessandra Eustáquio’s studies</td>
</tr>
</tbody>
</table>

**TABLE II.14: Plasmids and cosmids produced in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVD1</td>
<td>1 kb <em>BamH</em>l/<em>HindIII</em> fragment of pAE-G4 into the same sites of pUWL201, cointaining novG (position 6720 - 7704 in GenBank entry AF170880); AmpR, ThioR.</td>
</tr>
<tr>
<td>pVD4</td>
<td>1.314 kb <em>PstI</em>-<em>Sph</em>l fragment of pVD7 into the same sites of pWHM3, containing novE and 599 bp upstream of its start codon; AmpR, ThioR.</td>
</tr>
</tbody>
</table>
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**II.5.2. PCR primers used for construction of plasmids**

**TABLE II.15: Primers used for construction of plasmids**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)(^a)</th>
<th>Restriction site</th>
<th>Positions (Accession number)</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>PnovE(_f)</td>
<td>CCGACGCCTTACCACACCACCG</td>
<td>---</td>
<td>4209 - 4228 (AF170880)</td>
<td>pVD7</td>
</tr>
<tr>
<td>PnovE(_r)</td>
<td>GTCAACGGCGCCGCCCTCACCACAG</td>
<td>---</td>
<td>5443 - 5462 (AF170880)</td>
<td>pVD10</td>
</tr>
<tr>
<td>P10(_novE_f)</td>
<td>AAAAAGCTCCGCACGCTTCACCACCG</td>
<td><strong>HindIII</strong></td>
<td>4209 - 4228 (AF170880)</td>
<td></td>
</tr>
<tr>
<td>P10(_novE_r)</td>
<td>AAAAAGCATTCGCTAAACCGCTCCGCCTCACCACCG</td>
<td><strong>SphI</strong></td>
<td>5443 - 5462 (AF170880)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Underlines letters represent restriction sites **HindIII** and **SphI**.

---

**pVD7**
PCR fragment comprising \(novE\) (position 4209 – 5461 in AF170880) in pGEM-T; Amp\(^R\)

**pVD10**
1.31 kb **HindIII-SphI** fragment of PCR product comprising \(novE\) (position 4209-5461 in AF170880) into the same sites of pAE12

**Cosmid**

**nov-VD1** From nov-BG1, \(novE\) replaced by the apramycin resistance cassette from pUG019, Kan\(^R\), Apra\(^R\)

**nov-VD2** From nov-VD1, apramycin resistance cassette excised by expression of FLP-recombinase; \(\Delta novE\) cosmid (Fig. III.1), Kan\(^R\)

**nov-VD3** From nov-AE10, \(novE\) replaced by the apramycin resistance cassette from pUG019, Kan\(^R\), Apra\(^R\)

**nov-VD4** From nov-VD3, apramycin resistance cassette excised by expression of FLP-recombinase; \(\Delta novE\Delta novG\) cosmid (Fig. III.8), Kan\(^R\)

**nov-VD6** From nov-BG1, region \(novD\) stop codon to \(novH\) start codon replaced by apra-tcp830 from pMS80 (Fig. III.8); Kan\(^R\), Apra\(^R\)

**nov-VD7** From nov-VD6, apramycin resistance cassette excised by expression of FLP-recombinase (Fig. III.8); Kan\(^R\)

**nov-VD8** From nov-BG1, intergenic region \(novP\_novQ\) replaced by apra-tcp830 from pMS80 (Fig. III.8); Kan\(^R\), Apra\(^R\)

**nov-VD9** From nov-VD7, intergenic region \(novP\_novQ\) replaced by apra-tcp830 from pMS80 (Fig. III.8); Kan\(^R\), Apra\(^R\)
II. MATERIALS AND METHODS

II.5.3. Primers used for RT-PCR experiments

**TABLE II.16: Primers used for RT-PCR experiments**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5´-3´)</th>
<th>Position</th>
<th>Target gene</th>
</tr>
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<tbody>
<tr>
<td>P_hrdB_RT_f</td>
<td>TCGGCCAGCACATCCCCGTAC</td>
<td>6367668 - 6367687</td>
<td>hrdB</td>
</tr>
<tr>
<td>P_hrdB_RT_r</td>
<td>TCGGTACCCTCGGGGCTCCTC</td>
<td>6368203 - 6368222</td>
<td></td>
</tr>
<tr>
<td>P_novE_RT_f</td>
<td>GACGCGACCCCCCGTTGACAG</td>
<td>4880 - 4889</td>
<td>novE</td>
</tr>
<tr>
<td>P_novE_RT_r</td>
<td>GCCTCCCTCCACCCGTTCGAG</td>
<td>5378 - 5397</td>
<td></td>
</tr>
<tr>
<td>P_novG_RT_f</td>
<td>CCAACAGCGCGCGATGAGGAG</td>
<td>6734 - 6753</td>
<td>novG</td>
</tr>
<tr>
<td>P_novG_RT_r</td>
<td>CGTGGGACGACGACCTCAGC</td>
<td>7173 - 7192</td>
<td></td>
</tr>
</tbody>
</table>

The GenBank accession number for the sequence reported is AF170880 (novobiocin cluster) and SCO5820 (hrdB).

II.5.4. Primers used for qRT-PCR experiments

**Table II.17: Primers used for qRT-PCR experiments**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5´-3´)</th>
<th>Position</th>
<th>Target gene</th>
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<tbody>
<tr>
<td>P_hrdB_qRT_f</td>
<td>TGACGCTGATGGTGTCAGTGC</td>
<td>6367864 - 6367882</td>
<td>hrdB</td>
</tr>
<tr>
<td>P_hrdB_qRT_r</td>
<td>GTGCCTTCCTGCTGTCGTC</td>
<td>6367969 - 6367987</td>
<td></td>
</tr>
<tr>
<td>P_novE_qRT_f</td>
<td>GATCCCGCGGGACCTCTCC</td>
<td>4963 - 4980</td>
<td>novE</td>
</tr>
<tr>
<td>P_novE_qRT_r</td>
<td>CCAGCCATCGCGCGATCC</td>
<td>5043 - 5060</td>
<td></td>
</tr>
<tr>
<td>P_novF_qRT_f</td>
<td>GGAATGATCGCGACATCCAT</td>
<td>5563 - 5582</td>
<td>novF</td>
</tr>
<tr>
<td>P_novF_qRT_r</td>
<td>CAGTGGACGGGAGGACTGGT</td>
<td>5628 - 5646</td>
<td></td>
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<tr>
<td>P_novG_qRT_f</td>
<td>GTCGTGCACCGGTGGGACC</td>
<td>6901 – 6918</td>
<td>novG</td>
</tr>
<tr>
<td>P_novG_qRT_r</td>
<td>ACACATCGCGCCGGGAGGC</td>
<td>7001 - 7018</td>
<td></td>
</tr>
<tr>
<td>P_novH_qRT_f</td>
<td>CGAAACGGCTGGCAGACGC</td>
<td>8048 – 8065</td>
<td>novH</td>
</tr>
<tr>
<td>P_novH_qRT_r</td>
<td>GCCACGGCAAGCCACCGCTG</td>
<td>8138 - 8156</td>
<td></td>
</tr>
<tr>
<td>P_novO_qRT_f</td>
<td>CGTCCTCAACTGCTCCTCTCA</td>
<td>17908 – 17927</td>
<td>novO</td>
</tr>
<tr>
<td>P_novO_qRT_r</td>
<td>GCCTGATGGTGACGCGCCAGC</td>
<td>18021 - 18039</td>
<td></td>
</tr>
<tr>
<td>P_novP_qRT_f</td>
<td>GCCGGGACGGGTGACGAG</td>
<td>18301 - 18318</td>
<td>novP</td>
</tr>
<tr>
<td>P_novP_qRT_r</td>
<td>TGCGTACGGCGGGCCAGTC</td>
<td>18391 - 18408</td>
<td></td>
</tr>
<tr>
<td>P_novQ_qRT_f</td>
<td>GTGTGGCTTCTACGCCCCTGA</td>
<td>19728 – 19746</td>
<td>novQ</td>
</tr>
</tbody>
</table>
The GenBank accession number for the sequence reported is AF170880 (novobiocin cluster), SCO5820 (*hrdB*) and AF205854 (*gyrB*<sup>R</sup>)

### II.5.5. Bacterial strains

**TABLE II.18: Bacterial strains of *E. coli* and *Streptomyces***

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> XL1Blue MRF'</td>
<td>General cloning host (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB lacIqZDM15 Tn10 (Tet&lt;sup&gt;R&lt;/sup&gt;)], Tet&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Stratagene</td>
</tr>
<tr>
<td><em>E. coli</em> ET 12567</td>
<td>Strain triply defective in DNA methylation (<em>dam</em>- <em>dcm</em>- <em>hsdM</em>&lt;sup&gt;-&lt;/sup&gt;), Tet&lt;sup&gt;R&lt;/sup&gt;, Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(MacNeil et al., 1992)</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512</td>
<td>Δ<em>redD</em> Δ<em>aactII-ORF4</em> SCP1&lt;sup&gt;+&lt;/sup&gt; SCP2&lt;sup&gt;−&lt;/sup&gt; (no production of actinorhodin, undecylprodigiosin, and methylenomycin)</td>
<td>(Floriano &amp; Bibb, 1996)</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-BG1)</td>
<td><em>S. coelicolor</em> M512 containing the novobiocin cluster and flanking DNA regions, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Eustáquio et al., 2004)</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-BG1)/pWHM3</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Thio&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-BG1)/pVD4</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Thio&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-AE10)</td>
<td><em>S. coelicolor</em> M512 containing a <em>novG</em>-defective novobiocin cluster, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Eustáquio et al., 2005b)</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-AE10)/pWHM3</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Thio&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Eustáquio et al., 2005b)</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-AE10)/pAE8</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Thio&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Eustáquio et al., 2005b)</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-AE10)/pVD4</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Thio&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-VD2)</td>
<td><em>S. coelicolor</em> M512 containing a <em>novE</em>-defective novobiocin cluster, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-VD2)/pWHM3</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Thio&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-VD2)/pVD4</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Thio&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
</tbody>
</table>
### II. MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Strain Details</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. coelicolor</em> M512 (nov-VD2)/pVD1</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Thio&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-VD2)/pAE8</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Thio&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-VD4)</td>
<td><em>S. coelicolor</em> M512 containing a <em>novE</em>,<em>novG</em>-defective novobiocin cluster, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M12 (nov-VD4)/pWHM3</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Thio&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-VD4)/pVD10</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt;, Thio&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-VD7)</td>
<td><em>S. coelicolor</em> M512 containing nov-BG1 with the region from <em>novD</em> stop codon to <em>novH</em> start codon replaced by apra-tcp830; Kan&lt;sup&gt;R&lt;/sup&gt;, Apra&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-VD8)</td>
<td><em>S. coelicolor</em> M512 containing nov-BG1 with the intergenic region <em>novP</em>-<em>novQ</em> replaced by apra-tcp830; Kan&lt;sup&gt;R&lt;/sup&gt;, Apra&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-VD9)</td>
<td><em>S. coelicolor</em> M512 containing nov-VD7 with the intergenic region <em>novP</em>-<em>novQ</em> replaced by apra-tcp830; Kan&lt;sup&gt;R&lt;/sup&gt;, Apra&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This thesis</td>
</tr>
<tr>
<td><em>S. coelicolor</em> M512 (nov-JH4)</td>
<td><em>S. coelicolor</em> M512 containing a Ω&lt;sup&gt;novH&lt;/sup&gt; novobiocin cluster; Kan&lt;sup&gt;R&lt;/sup&gt;, Apra&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Johannes Härle’s Thesis</td>
</tr>
</tbody>
</table>

#### II.5.6. Probe used in Southern blot analysis

Cosmid nov-BG1 after digestion with *Pst*I and DIG-labelling, was used as probe for Southern blot analysis.

#### II.6. Culture conditions

#### II.6.1. Cultivation of *E. coli*

For cloning experiments, *E. coli* strains were grown overnight (16-18 h) in liquid or solid LB medium with appropriate antibiotic(s) at 37 °C (Sambrook & Russell, 2001). Permanent cultures of *E. coli* were prepared by mixing 600 µl of overnight culture with 400 µl of glycerol solution (50% (w/v) in distilled water) and stored at -70°C.
II.6.2. Cultivation of *Streptomyces coelicolor*

II.6.2.1. General cultivation

*Streptomyces coelicolor* strains were routinely cultured in liquid YMG medium. These cultures were carried out by inoculation of $10^8$ spores into 300 ml baffled Erlenmeyer flasks containing a stainless steel spring and 50 ml YMG medium and cultivated at 200 rpm and 30 °C for 48 hours. For preparation of protoplasts, *S. coelicolor* was cultured in TSB medium containing 0.4% glycine. For isolation of genomic DNA, *S. coelicolor* was cultured in TSB medium. An appropriate concentration of antibiotic(s) was added, if required.

II.6.2.2. Production of secondary metabolites

For production of secondary metabolites *S. coelicolor* strains containing the entire or modified novobiocin biosynthetic gene cluster were cultivated in 300 ml baffled Erlenmeyer flasks or 24-square deepwell plates.

**Note:** Cultivation of *Streptomyces coelicolor* strains in 24-square deepwell plates (Duetz *et al.*, 2000) resulted in higher reproducibility of cell growth and novobiocin production in comparison to conventional Erlenmeyer flask cultivations. Therefore, cultivation in 24-square deepwell plates are recommended especially for quantitative analysis of secondary metabolite production and (q)RT-PCR experiments.

**Cultivation in Erlenmeyer flasks**

For production of secondary metabolites, 1ml of the YMG preculture of *S. coelicolor* containing the entire or modified novobiocin gene cluster was inoculated into 300 ml baffled flasks containing a stainless steel spring and 50 ml CDM production medium and cultivated at 30 °C and 200 rpm for 7 days. The cultivation in production medium was carried out without addition of antibiotics.
**Cultivation in 24-square deepwell plates**

For cultivation in 24-square deepwell plates frozen and homogenized inoculum of *Streptomyces coelicolor* strains were prepared as described in II.6.2.3. $10^6$ CFU of this inoculum was mixed with 40 ml CDM production medium, containing 0.6 % (m/v) siloxylated polyether EO/PO copolymer Q2-5247 (Dow Corning, Auburn, Michigan/USA) and 3 ml of this mixture were placed into each well of the 24-square deepwell plates (Duetz et al., 2000). Cultivation was carried out at 30 °C and 300 rpm for 7 days. This cultivation was carried out without addition of antibiotics.

**II.6.2.3. Preparation of homogenized and frozen inoculum**

For preparation of frozen inoculum, 50 ml of YMG preculture were centrifuged (2772 x g for 10 min). The cells were resuspended in 10 ml of an aqueous solution of 20 % (w/v) peptone (Bacto® Proteose Peptone Nr. 3, Difco, Sparks, Maryland/USA) and gently homogenized using a potter homogenizer operated manually (B. Braun Biotech, Sartorius AG, Göttingen, Germany). The resulting mixture was divided in aliquots and stored at -70 °C.

**II.6.2.4. Preparation of mycelia for storage and spore suspensions of *Streptomyces***

For preparation of mycelia for storage, 1 ml 2-day-old YMG culture was harvested by centrifugation and the cells were resuspended in 0.5 ml 20 % glycerol. The storage was carried out at -70°C.

To prepare spore suspensions, *Streptomyces coelicolor* strains were spread on MS agar and incubated at 30 °C for about one week. The plates (one to two plates for good sporulaters, four for more sparsely sporulating strains) were grown till they were well sporulated. 4 ml of Tween® 20 (0.1% (w/v)) were added to each plate and the spores scraped off of the top of the plates and into suspension. The resulting spore suspension was poured into a falcon tube and vortexed vigorously (about 1 min). The spores were separated from the mycelium by passing the suspension through sterile cotton plugged in a disposable syringe. Spores were collected by centrifugation
II. MATERIALS AND METHODS

(2,100×g, 10 min, 4 °C), and resuspended in 1-3 ml of 20% glycerol. The spore suspensions were kept at -70 °C.

Note: For selection of Streptomyces coelicolor strains with thiostreptone as selection marker, strains were spread on MM agar!

II.7. Methods of molecular biology

II.7.1. Purification, concentration and quantification of DNA

Standard methods for DNA isolation and manipulation were performed as described elsewhere (Kieser et al., 2000; Sambrook & Russell, 2001).

Phenol/chloroform extraction and ion exchange column chromatography were used for purification of DNA. Ethanol or isopropanol precipitation was used for concentration.

Quantification of DNA was carried out by using a GeneQuant photometer (Pharmacia, Freiburg, Germany) at 260 nm as well as by comparing the fluorescent intensity with DNA markers on agarose gels.

II.7.2. Agarose gel electrophoresis of DNA

Gel electrophoresis with 0.8-1.5% (w/v) agarose was used to separate DNA fragments between 0.5 and 50 kb, and with 2-2.5% NuSieve®GTG® agarose to separate DNA fragments between 0.1 and 0.5 kb. The buffer system employed was 1×TAE buffer (Table II.8). After running the gels, they were stained with the fluorescent dye ethidium bromide, detected under the UV light at 312 nm and photographed by using Eagle Eye II System (Strategene, Heidelberg, Germany) (Sambrook & Russell, 2001).

DNA fragments were isolated from agarose gels using a NucleoSpin® 2 in 1 extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol.
II.7.3. DNA manipulation with enzymes

Restriction of DNA with endonucleases was carried out according to the manufacturer's instructions.

DNA ligation was achieved by using T4-DNA ligase. The ligation preparation, containing 1U T4 DNA ligase, 1× ligation buffer and a 1:1 (mole ratio) mixture of insert and linearized vector (about 100 ng) in a final volume of 10 µl, was incubated at room temperature for 2 h or at 16 °C or 4 °C overnight.

II.7.4. DNA isolation

II.7.4.1. Isolation of plasmids from *E. coli*

Mini-preps employing alkaline lysis were used to isolate recombinant plasmids from *E. coli* for routine screening. 3 ml LB-medium was inoculated with a single colony and grown overnight at 37 °C, 170 rpm. 2 ml of this culture were harvested by centrifugation (10000×g, 4 °C, 1 min) and resuspended in 250 µl solution MP1 by vortexing. The suspension was mixed with 250 µl solution MP2 by inversion and incubated at room temperature for 1-5 min. 250 µl solution MP3 was added and the mixture was incubated on ice for 5 min. After centrifugation (20,000×g, 4 °C, 15 min), the supernatant was poured into a fresh microfuge tube. The DNA was precipitated by addition of 0.8-fold isopropanol and centrifugation (20,000×g, 4 °C, 30 min). The DNA pellet was washed with 500 µl 70% ethanol, air dried and resuspended in 50 µl distilled water or TE buffer. The mentioned solutions are listed in Table II.5.

Preparative isolation of plasmids from *E. coli* was carried out with ion-exchange columns (Nucleobond® AX100, Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol.

II.7.4.2. Isolation of plasmids from *Streptomyces*

Isolation of plasmid DNA from *Streptomyces* strains was carried out by alkaline lysis and potassium acetate precipitation, adapted from procedure D (Kieser *et al.*, 2000). 2 ml of a 2-day-old culture in YMG medium were harvested by centrifugation (17,000×g, 4 °C, 1 min). After washing with 1ml of solution MP1, the cells were resuspended in 500 µl of solution MP1GL by vortexing. The suspension was
incubated at 37 °C for 30-60 min, then mixed with 500 µl of solution MP2 by inversion and incubated at room temperature for 10 min. 400 µl solution MP3 and 40 µl Rotiphenol® were added and mixed by inversion. The mixture was incubated on ice for 5 min. After 20 min centrifugation (20,000×g, 4 °C), the supernatant was poured into a fresh microfuge tube and extracted twice with 300 µl Phenol/Chloroform/Isoamylalcohol (25:24:1). The DNA was precipitated by addition of 0.8-fold volume of isopropanol and centrifugation (20,000×g, 4 °C, 20 min). The DNA pellet was washed with 500 µl 70% ethanol, air dried and resuspended in 20 - 50 µl distilled water or TE buffer. If required, the plasmid DNA isolated from *Streptomyces* was amplified in *E. coli* XL1 blue MRF⁻ before restriction analysis. The mentioned solutions are listed in Tables II.5 and II.6.

**II.7.4.3. Isolation of genomic DNA from *Streptomyces coelicolor***

Genomic DNA was isolated by the Kirby mix procedure (Kieser *et al*., 2000). 2 ml of a 2-day-old culture in TSB medium were harvested by centrifugation (17,000×g, 4 °C, 1 min). The cells were washed with 1 ml TSE buffer and resuspended in 500 µl TSE buffer with lysozym (3 mg/ml) and RNase A (100 µg/ml) by vortexing. The suspension was incubated for 15 min at 37 °C. 400 µl of 2×Kirby mix were added and the mixture was vortexed vigorously for 1 min. 800 µl phenol/chloroform/isoamyl alcohol (25:24:1) were added and the mixture was vortexed vigorously for 15 s and centrifuged at 17,000×g and 4 °C for 10 min. The supernatant was poured into a fresh microfuge tube and extracted a second time with addition of 70 µl “unbuffered” 3 M NaOAc and 300 µl phenol/chloroform/isoamyl alcohol (25:24:1) (1 min vigorous vortexing). The aqueous phase was separated and genomic DNA was precipitated by addition of 0.8-fold volume of isopropanol and centrifugation (20,000×g, 4 °C, 30 min). The DNA pellet was washed with 500 µl 70% ethanol, air dried and resuspended in 50 to 100 µl TE buffer.
II.7.5. DNA denaturation by alkaline treatment for ssDNA transformation in \textit{Streptomyces}

9 µl dsDNA in H$_2$O was mixed with 2 µl 1 M NaOH and incubated for 10 min at 37 °C. The mixture was placed on ice and the reaction was terminated with addition of 2 µl 1 M HCl (Oh & Chater, 1997).

II.7.6. PCR amplification

II.7.6.1. General conditions

PCR amplifications were carried out with the GeneAmp\textsuperscript{®} PCR System 2400 or GeneAmp\textsuperscript{®} PCR-System 9700 (Perkin-Eimer, Weiterstadt, Germany) or iCycler PCR-System (Bio-Rad, Munich, Germany). The amplification conditions for PCR reactions using the Expand High Fidelity PCR system (Roche) are given in Table II.19, and using the GC-Rich PCR system (Roche) in Table II.20.

\textbf{TABLE II.19: PCR reaction and amplification conditions using the Expand High Fidelity PCR system}

<table>
<thead>
<tr>
<th>Substance</th>
<th>Final concentration</th>
<th>Cyclus</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction buffer (10×)</td>
<td>1×</td>
<td>Hot start</td>
<td>94 °C</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>DMSO</td>
<td>5% (v/v)</td>
<td>Denaturing</td>
<td>94 °C</td>
<td>45 s</td>
<td>30</td>
</tr>
<tr>
<td>Template DNA</td>
<td>about 100 ng</td>
<td>Annealing</td>
<td>50-60 °C</td>
<td>45 s</td>
<td></td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.2 mM each</td>
<td>Elongation</td>
<td>72 °C</td>
<td>90 s</td>
<td></td>
</tr>
<tr>
<td>Primer</td>
<td>50 pmol each</td>
<td>Final elongation</td>
<td>72 °C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>DNA-Polymerase</td>
<td>2.5 U</td>
<td>End</td>
<td>4 °C</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

Add distilled water to make up to 50 µl
TABLE II.20: PCR reaction and amplification conditions using the GC-Rich PCR system

<table>
<thead>
<tr>
<th>Substance</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC-Rich PCR reaction buffer with DMSO (5×)</td>
<td>1×</td>
</tr>
<tr>
<td>GC-Rich resolution solution (5 M)</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>dNTP-Mix</td>
<td>0.2 mM each</td>
</tr>
<tr>
<td>Primer 1</td>
<td>20 pmol</td>
</tr>
<tr>
<td>Primer 2</td>
<td>20 pmol</td>
</tr>
<tr>
<td>Template DNA</td>
<td>about 100 ng</td>
</tr>
<tr>
<td>GC-Rich PCR System enzyme mix</td>
<td>2 U</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substance</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot start</td>
<td>95 °C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturing</td>
<td>95 °C</td>
<td>90 s</td>
<td>30</td>
</tr>
<tr>
<td>Annealing</td>
<td>55-60 °C</td>
<td>90 s</td>
<td></td>
</tr>
<tr>
<td>Elongation</td>
<td>72 °C</td>
<td>45s/1 kb</td>
<td></td>
</tr>
<tr>
<td>Final elongation</td>
<td>72 °C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>End</td>
<td>4 °C</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

Add distilled water to make up to 100 µl

For PCR amplification with *Taq* or *Pfu* polymerase, the PCR mixture (50-100 µl) contained 50 pmol each primer, 100-300 ng template DNA, 0.2 mM each dNTP, 1× reaction buffer, 5% (v/v) DMSO and 2-3 U polymerase. Amplification conditions were according to the supplier's instructions.

In general, 5 to 10 µl of the PCR reaction were analysed by gel electrophoresis.

II.7.6.2. Conditions for amplification of the apramycin resistance cassette from pUG019 and the apra-tcp830 cassette from pMS80

The conditions for amplification of the apra-tcp830 from pMS80 (REDIRECT© technology kit for PCR targeting (Gust et al., 2003)) using the Expand High Fidelity PCR system (Roche) are given in Table II.21. Template DNA was prepared by digesting about 10 µg of pMS80 with *KpnI* and *SacII*, and by isolating the 1.7 kb cassette fragment from an agarose gel (Gust et al., 2003).
TABLE II.21: Conditions for amplification of the apramycin resistance cassette from pUG019 and apra-tcp830 from pMS80

<table>
<thead>
<tr>
<th>Substance</th>
<th>Final concentration</th>
<th>Cyclus</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction buffer (10×)</td>
<td></td>
<td>Hot start</td>
<td>94 °C</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>DMSO</td>
<td>5% (v/v)</td>
<td>Denaturing</td>
<td>94 °C</td>
<td>45 s</td>
<td>10</td>
</tr>
<tr>
<td>Template DNA</td>
<td>about 100 ng</td>
<td>Annealing</td>
<td>50 °C</td>
<td>45 s</td>
<td></td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.2 mM each</td>
<td>Elongation</td>
<td>72 °C</td>
<td>90 s</td>
<td></td>
</tr>
<tr>
<td>Primer</td>
<td>50 pmol each</td>
<td>Denaturing</td>
<td>94 °C</td>
<td>45 s</td>
<td>15</td>
</tr>
<tr>
<td>DNA-Polymerase</td>
<td>2.5 U</td>
<td>Annealing</td>
<td>55 °C</td>
<td>45 s</td>
<td></td>
</tr>
<tr>
<td>Add distilled water to make up to 50 µl</td>
<td>Elongation</td>
<td>72 °C</td>
<td>90 s</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Final elongation</td>
<td>72 °C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>End</td>
<td>4 °C</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

For amplification of the apramycin resistance cassette from pUG019 the conditions were the same as listed in Table II.21. Template DNA was prepared by digesting about 2 µg of pUG019 with EcoRI, HindIII and Dral (Dral cuts three times in the vector backbone, and it is used to guarantee the destruction of traces of cccDNA and to avoid purification of the cassette by gel electrophoresis). After digestion, the mixture was purified and concentrated by isopropanol precipitation.

II.7.7. Southern blot analysis

Southern blot analysis was performed on Hybond-N membranes (Amersham) by using the DIG high prime DNA labelling and detection starter kit II (Roche). Buffers and solutions are given in Table II.9.

Note: powder-free gloves were always worn to handle the used plastic or glass ware and the membrane (which should, if possible, only be handled with a pair of clean tweezers on the edges). Plastic and glass ware were thoroughly washed with distilled water before use.
II. MATERIALS AND METHODS

II.7.7.1. Probe preparation

Probe for Southern hybridization was prepared by the random priming method using the DIG high prime DNA labelling and detection starter Kit II (Roche) according to the user’s manual. The probe used in this thesis is presented in section II.5.6.

II.7.7.2. Southern blot preparation

An agarose gel with DNA digested with appropriated enzymes and the DIG Molecular Weight Marker VII (Roche) was run. The gel was stained with ethidium bromide and photographed. The DNA was denaturized by soaking the gel in denaturing buffer for $2 \times 15$ min, and then neutralized by soaking in neutralizing buffer for $2 \times 15$ min, with gentle agitation. Southern blot was carried out by capillary transfer using $20 \times$ SSC buffer. For this purpose, the gel was placed on pre-wetted (in $20 \times$ SSC buffer) filter paper and overlaid with pre-wetted (in $2 \times$ SSC buffer) Hybond-N nylon membrane and pre-wetted (in $20 \times$ SSC buffer) filter paper. About 7 cm paper towels and a 1 kg weight were stacked on the top. The transfer was carried out overnight. The membrane was then crosslinked with UV light (312 nm, 60 s on the front side and 30 s on the backside) and washed with sterile, distilled water. The membrane was used immediately or stored at 4 °C after air drying.

II.7.7.3. Prehybridization and hybridization

The membrane was incubated in prehybridization solution ($20 \text{ ml}/100 \text{ cm}^2$) for 4 h at 68 °C with gentle rotation. Appropriate probe was added to the hybridization solution (5-25 ng/ml). This was denaturized by heating (10 min in a boiling water bath), and immediately chilled in liquid nitrogen. The prehybridization solution was replaced by the hybridization solution containing the appropriate probe (about 7 ml/100 cm$^2$) and incubated overnight at 68 °C.

II.7.7.4. Detection

The membrane was washed twice with $2 \times$ washing buffer for 10 min at room temperature, and afterwards twice with $0.5 \times$ washing buffer at 68 °C for 20 min, with gentle rotation. After equilibration in maleic acid buffer for 5 min, the membrane was incubated with blocking solution for 30 min and then with antibody solution for 30 min.
To remove excess of antibody, the membrane was washed twice in Tween® washing buffer, 15 min each at room temperature. Finally, the membrane was equilibrated in detection buffer for 5 min and immediately placed between two plastic sheets (which were sealed in two sides) with the backside of the membrane placed on one sheet, and the front side remaining free by lifting the second sheet. A 1:100 dilution of CSPD stock solution in detection buffer was dropped onto the membrane (0.5 ml/100cm²) and spread over it by letting down the second sheet. It was incubated at room temperature for 2-5 min protected from light. Excess of solution and air bubbles were removed by wiping the cover sheet with a clean paper towel. The membrane was exposed to a Hyperfilm ECL-X-ray film (Amersham Biosciences, Freiburg, Germany) at 37 °C for 30 min to 3 h, depending on the strength of the signal. The film was developed using standard methods.

II.7.7.5. Removal of probe

If necessary, probe was removed by washing the membrane twice with stripping solution for 15 min at 37 °C, followed by washing with 2×SSC solution for 30 min at room temperature. After that, the membrane could be used for further hybridization or stored in 2×SSC solution at 4 °C.

II.7.8. Introduction of DNA in E. coli

The methods described in this section were modified from (Sambrook & Russell, 2001).

II.7.8.1. CaCl₂-mediated transformation

_Preparation of competent cells:_ 100 ml LB-medium were inoculated with 1 ml of an overnight culture of _E. coli_ and cultivated at 37 °C and 170 rpm till the OD₆₀₀ reached 0.6 (2.5-4 h). The cells were harvested by centrifugation (3,000×g, 4 °C, 5 min), resuspended in 30 ml ice-cold 0.1 M MgCl₂ and again centrifuged as above. The cell pellet was suspended in 20 ml ice-cold CaCl₂ (0.1 M) and incubated on ice for 20 min. After centrifugation, the pellet was suspended in 5 ml of CaCl₂ (0.1 M) solution containing 15% glycerol. Competent cells could be used immediately or dispensed in 200 µl aliquots in 1.5-ml microfuge tubes, and stored at -70 °C.
Note: Resuspension of cells should not be done by vortexing. Therefore, it is easier and quicker to resuspend the cells first in the remaining drops (after discarding the supernatant) by tapping the tube, and only afterwards to add the required solution and mix gently by inversion.

**Transformation:** DNA (0.1-1 µg in 1-5 µl) was added to 100-200 µl competent cells in 1.5-ml microfuge tube and incubated on ice for 30 min. The tube was then incubated at 42 °C for 2 min and cooled down on ice (about 5 min). 1 ml LB medium was pipetted into the tube, and the suspension was incubated on a water bath or on a shaker (170 rpm) for 1 h at 37 °C. 200 µl of the mixture were spread on a LB agar plate containing the appropriate antibiotic(s) and the rest was centrifuged (17,000×g, 4 °C, 30 s), resuspended in 200 µl LB and spread on another LB agar plate. The plates were incubated at 37 °C. For transformation of *E. coli* XL1 Blue MRF' cells with a circular plasmid (cccDNA), the incubation on ice might be shortened to 10 min and the incubation with LB medium at 37 °C might be omitted, since the transformation efficiency is otherwise too high to allow growth of single colonies. However, for *E. coli* ET12567, all the procedure as described above should be carried out, as the transformation efficiency of this strain is lower (about 100-fold).

**Blue/white selection:** If a *lacZα*-containing cloning vector was used to prepare the recombinant plasmid, blue/white selection can facilitate the identification of the expected clones. For this purpose, first 15 µl of IPTG solution (80 mg/ml) in up to 100 µl H₂O (sterile) were pipetted on the top of the plates and spread evenly, and then 60 µl of X-Gal solution (20 mg/ml in DMF) was plated in the same way. The plates were air dried under the laminar flow for 30-45 min in order to evaporate the toxic DMF. Colonies containing the recombinant plasmid lack β-galactosidase activity and remain white.

II.7.8.2. Electroporation

**Preparation of electro-competent cells:** 50 ml LB-medium was inoculated with 1 ml of an overnight culture of *E. coli* and cultivated at 30 to 37 °C (see note below), 170 rpm till the OD₆₀₀ reached 0.6 (2.5-4 h). The cells were harvested by centrifugation (3,000×g, 4°C, 5 min), and washed twice with 50 and 25 ml ice-cold 10% (w/v) glycerol solution, respectively. The cell pellet was suspended in the remaining drops
after discarding the supernatant. Competent cells could be used immediately or dispersed in 50-µl aliquots in 1.5-ml microfuge tubes, and stored at -70 °C.

**Electroporation**: DNA (about 100 ng in 1-2 µl distilled water) was added to 50 µl competent cells in 1.5-ml microfuge tube and incubated on ice for about 1 min. The mixture was then carefully transferred to an ice-cold electroporation cuvette (0.2 cm), avoiding formation of air bubbles, and electroporation was carried out using a BioRad electroporator set to 2.5 kV. The optimal time constant is 4.5 – 5.0 ms. 1 ml LB medium was immediately pipetted into the cuvette, and the suspension was transferred to a microfuge tube and incubated on a water bath or on a shaker (170 rpm) for 1 h at 30 to 37 °C. The mixture was spread on LB agar plates containing the appropriate antibiotic(s) (no more than 200 µl per plate) and the plates were incubated at 30 to 37 °C.

**Note**: To maintain plasmid pIJ790 in *E. coli* BW25113 (REDIRECT© technology kit for PCR targeting (Gust et al., 2003)), cells must be cultured at 30 °C, since pIJ790 contains a temperature sensitive origin of replication. Otherwise, the cultivation temperature was 37 °C.

**II.7.9. PEG-mediated protoplast transformation for introduction of DNA in Streptomyces**

Relevant buffers and media are listed in Table II.11 and in section II.4.1.4, respectively.

**Preparation of protoplasts from S. coelicolor**

Slightly modified from (Kieser et al., 2000). Mycelium from a 40 h old culture (50 ml TSB medium containing 0.4 % glycine) was washed twice with 15 ml of a 10.3% sucrose solution, resuspended in 10 ml of lysozyme solution (2 mg/ml in P buffer) and incubated at 30 °C for 15-60 min with gentle agitation. Protoplast formation was monitored using the microscope. After most cells became protoplasts, the reaction was stopped by incubation on ice. The following steps were carried out on ice. 10 ml of ice-cold P buffer were added and the suspension was drawn in and out of a 10 ml pipette three times and filtered through glass wool. Protoplasts were sedimented
II. MATERIALS AND METHODS

II.7. gently by centrifugation (e.g. 1,000xg, 7 min). The supernatant was discarded, the
pellet was first carefully resuspended in the remaining drop of liquid by tapping the
tube, and then in 1 ml P buffer. The protoplast suspension can be immediately used
for transformation or 100 µl aliquots can be stored at -70 °C. To freeze protoplasts for
storage, tubes were placed in ice contained in a plastic beaker, and the beaker was
placed at -70 °C overnight. To assess the protoplast regeneration, dilution series of
the protoplast suspension in P buffer were prepared and plated on R5 agar plates
(see note below). The plates were incubated at 30 °C for 3-7 days. The regenerable
protoplasts per ml suspension were calculated. To assess the proportion of non-
protoplasted units in the suspension, samples were also diluted in distilled water and
plated on regeneration plates (R5 agar).

**Note**: plating of protoplasts was always done by overlaying with R5 soft agar instead
of spreading in order to avoid mechanical stress and lysis.

**Transformation**: The transformation of *Streptomyces* strains was carried out by a
modification of the method described by Kieser *et al.* (2000).

Before transformation of *Streptomyces* strains the plasmids were propagated in *E.
coli* ET 12567 to bypass methyl-sensing restriction.

1-20 µg DNA (in maximal 10-20 µl TE buffer) were added to 100-200 µl of a
protoplast suspension, containing at least 10^8 protoplasts per ml; 400-500 µl T-buffer
containing PEG 1000 (25% (w/v) were immediately added, mixed by pipetting
carefully three times and incubated at room temperature for 1 min. Increasing
volumes of the resulting suspension (e.g. 10 µl, 100 µl, 200 µl, rest) were mixed with
warm R5 soft agar (about 50 °C, 4×3 ml) and plated on four R5 plates. After 16-24 h
incubation at 30 °C, the plates were overlaid with 3 ml of R5 soft agar including the
required antibiotics for selection of mutants, and incubation was continued for further
3-7 days.

II.7.10. DNA sequencing and computer-assisted sequence analysis

Double-stranded sequencing of recombinant plasmids was done by the
dideoxynucleotide chain termination method on a LI-COR automatic sequencer
(MWG-Biotech AG, Ebersberg, Germany).
The DNASIS software package (Version 2.1, Hitachi Software Engineering, San Bruno, CA, USA) was used for sequence analysis. Amino acid sequence homology searches were performed by using the BLAST program (Version 2.0) available on the web at [www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/).

II. MATERIALS AND METHODS

II.8. RNA methods

II.8.1. RNA isolation, DNase treatment and purification

For RT-PCR experiments *Streptomyces coelicolor* M512 strains were cultivated in 300 ml baffled Erlenmeyer flasks as described above. From each mutant 1 ml of YMG preculture was inoculated into a 300 ml baffled flask containing 50 ml CDM production medium (Kominek, 1972) and cultivated at 30 °C and 200 rpm. After 22, 30, 44, 77 and 94 hours of cultivation the mycelia of 50 ml culture was collected by vacuum filtration, followed by rinsing with 30 ml distilled water.

**Note:** Cultivation of *Streptomyces coelicolor* M512 strains for RT-PCR experiments were carried out in Erlenmeyer flasks because at this time point the cultivation procedure in 24-square deepwell plates was not established. Everyone working on regulation projects is recommended to carry out cultivation in 24-square deepwell plates (see below) due to its very stable and reproducible cell growth and production!

For qRT-PCR experiments *Streptomyces coelicolor* strains were cultivated in 24-square deepwell plates (Duetz *et al.*, 2000) as described above. From each mutant 2 x 10^6 CFU of frozen inoculum were mixed with 80 ml CDM production medium, containing 0.6 % (m/v) siloxylated polyether EO/PO copolymer Q2-5247 (Dow Corning, Auburn, Michigan/USA) and 3 ml of this mixture were placed into 27 wells of 2 x 24-square deepwell plates. Cultivation was carried out at 30 °C and 300 rpm. After 24, 32, 48, 72 and 96 hours mycelia of 6 to 4 wells were pooled and collected by vacuum filtration, followed by rinsing with 30 ml distilled water. After 168 hours 3 wells were harvested to analyze final novobiocin production.

Then the cells were transferred to a universal plastic tube (50 ml falcon tube) containing approximately 14 g 3.5 – 4.5 mm diameter glass beads and 15 ml of modified kirby mix Table II.12 and vortexed for 2 min. After that, cell suspension was
sonicated for 6 x 30 s with 20 s intervals between each sonication treatment (Branson sonifier 250). To this cell suspension 1 volume (15 ml) of phenol/chloroform/isoamylalcohol (25:24:1) was added and followed by vortexing for 30 s and centrifugation (5000 x g for 10 min at 4 °C).

**Note:** Due to the instability of RNA the following steps have to be carried out carefully to avoid contamination with RNase. Therefore, gloves were always worn to handle the used plastic or glass ware. Plastic, glass ware, water and solutions (except from ethanol, isopropanol, modified kirby mix and phenol/chloroform/isoamylalcohol 25:24:1) were autoclaved twice before use. Furthermore, breezing and talking into probes was avoided.

Clear supernatant was transferred to a clean tube (50 ml falcon tube) and mixed with an equal volume of isopropanol and 0.1 volume 3 M sodium acetate (pH: 5.2) and leaved for 5 min at 20 °C. After centrifugation (5000 x g for 20 min at 4 °C) supernatant was discarded and the pellet was washed with 2 ml ethanol 70 % (v/v). Then pellet was dried and resuspended in 800 µl water. After resuspension DNase treatment was carried out, using Deoxyribonuclease I (1U µl⁻¹), 10 x buffer with MgCl₂ (100 mM Tris-HCl (pH 7.5 at 25 °C, 25 mM MgCl₂, 1mM CaCl₂) and RNase-free water (Fermentas) according to manufacturer’s instructions. After that, RNA was purified with the NucleoSpin® RNA Clean-up Kit (Macherey & Nagel) according to manufacturer’s instruction and eluted from the column with 40 µl water, followed by its quantification by determining the OD at 280 nm. Integrity of RNA was checked by running 1 µg of RNA on a 1.2 % agarose gel. To ensure that RNA is free of DNA, PCR using RNA-probes without previous RT-reaction (PCR-conditions and primers as presented below; concentration tenfold to the concentration that was used in RT-reaction) was carried out.

**II.8.2. RT-PCR**

After ensuring that RNA is free of DNA the RT-reaction was carried out. Therefore random hexamer primers (0.2 µg µl⁻¹), 5 x reaction buffer (250 mM Tris-HCl (pH: 8.3 at 25 °C), 250 mM KCl, 20 mM MgCl₂, 50 mM DTT), RiboLock™ Ribonuclease Inhibitor (20U µl⁻¹), 10 mM dNTP mix (10 mM each), RNase-free water and
RevertAid™M-MuLV Reverse Transcriptase (200U µl⁻¹) (Fermentas) were used. RT-reaction was carried out according to the Fermenta’s “Protocol for First Strand cDNA Synthesis”.

After RT-reaction PCR using primer pairs for amplification of hrdB, novE and novG listed in Table II.16 was carried out in 50 µl volume with 2 µl template (cDNA from RT-reaction), 2.5 mM dNTPs each, 50 pmol each primer, 5 % (v/v) DMSO, 10 x buffer (100mM Tris-HCl, pH: 8.8 at 25 °C; 500 mM KCl, 0.8 % (v/v) Nonidet P40 and 15 mM MgCl₂) and 1 µl Taq DNA-polymerase (1U µl⁻¹): denaturation at 95 °C for 2 min; 27 cycles with denaturation at 95 °C for 30 s, annealing at 70 °C for 30 s and extension at 72 °C for 50 s, and a final elongation step at 72 °C for 7 min. Finally PCR product was checked by running 22 µl of the PCR-reaction with 8 µl loading buffer [50% glycerol, 200mM EDTA, 0.5 % xylene cyanol(Sigma®)] on a 1.2 % agarose gel.

II.8.3. qRT-PCR
LightCycler® quantitative RT-PCR (qRT-PCR) was carried out using the LightCycler® RNA amplification Kit SYBR Green I (Roche). Master mixes were prepared by following the manufacturer’s instructions for GC-rich templates except from [MgCl₂] that was reduced to 6.25 mM for investigations on novE, novF, novG, novH, novO, novQ and gyrBR and 12.5 mM for investigations on hrdB and novP, using the primers listed in Table II.17. After RT for 20 min at 50°C, the following temperature profile was utilized for amplification: denaturation for 1 cycle at 95 °C for 30 s and 45 cycles at 95 °C for 1 s (temperature transition, 20 °C/s), 60 to 55 °C (novE, novG, novH, novO), 58 to 53 °C (hrdB, novQ), 56 to 52 °C (novF, novP, gyrBR) (step size, 0.7°C; step delay, 1 cycle) for 10 s (temperature transition, 20 °C/s), and 72 °C for 13 s (temperature transition, 2 °C/s) with stepwise fluorescence acquisition at 60 to 55 °C in single mode. The number of copies of each sample transcript was then determined with the aid of LightCycler® software and normalised to hrdB. The specificity of the PCR reaction was verified by ethidium bromide staining on 2% agarose gels.
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II.9. Heterologous expression of the modified novobiocin biosynthetic gene clusters

II.9.1. Inactivation of novE in cosmids nov-BG1 and nov-AE10, and heterologous expression of the ΔnovE and ΔnovEΔnovG cosmid

The gene novE in cosmid nov-BG1 and cosmid nov-AE10 (ΔnovG cosmid) (Eustáquio et al., 2005b) was replaced by the apramycin resistance (aac(3)IV) cassette from pUG019 (Eustáquio et al., 2004) via λ-Red-mediated recombination (Gust et al., 2003) resulting in cosmid nov-VD1 (from nov-BG1) and nov-VD3 (from nov-AE10). This cassette is flanked by the FRT (FLP recognition target). The cassette for replacement of novE was generated by PCR using the primer pair P1_novE (5´-CGC CGG TCC GCT TGT CCC GAG GGG AAG AGA GGC ATC GTG ATT CCG GGG ATC CGT CGA CC-3´) and P2_novE (5´-GCC GTG AGG CCG CGA AAT GGA TCG GAG TGC GTC CGG TCA TGT AGG CTG GAG CTG CTT C-3´). Bold letters respresent 39 nt homologous extensions to the DNA regions immediately upstream and downstream of novE, including the putative translational start and stop codons of novE, respectively. The PCR reaction was performed in 50 µl volume with 50 ng template (pUG019 digested with EcoRI, HindIII and Dral), 0.2 mM dNTPs, 50 pmol each primer, 5 % (v/v) DMSO, using the Expand High Fidelity PCR System (Roche Molecular Biochemicals): denaturation at 94 °C for 2 min, then 10 cycles with denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 90 s, then 15 cycles with annealing at 55 °C, and a final elongation step at 72 °C for 5 min. Subsequently, the cassette was excised by the FLP recombinase, leaving an in-frame “scar” of 81 nucleotides between the start and stop codon of novE, resulting in cosmid nov-VD2 (ΔnovE) and nov-VD4 (ΔnovEΔnovG). E. coli XL1 Blue MRF’ cells were analysed using restriction enzymes and gel electrophoresis. The generated ΔnovE cosmid (nov-VD2) and ΔnovEΔnovG cosmid (nov-VD4), carrying the kanamycin resistance gene neo, were then introduced into S. coelicolor M512 by PEG-mediated protoplast transformation (Kieser et al. 2000). Kanamycin resistant clones were checked for specific genomic integration of cosmid nov-VD2 or cosmid nov-VD4 into the ΦC31 attachment site by Southern blot analysis.
II. MATERIALS AND METHODS

II.9.2. Introduction of tcp830 into cosmid nov-BG1 and heterologous expression of the resulting cosmids

The apramycin-tcp830 cassette (apra-tcp830) from pMS80 (Rodriguez-Garcia et al., 2005) was introduced in nov-BG1 via λ-Red-mediated recombination (Gust et al., 2003), either in exchange with the region from novD stop codon to novH start codon, resulting in cosmid nov-VD6 or by replacing the intergenic region novP_novQ, resulting in cosmid nov-VD8. The apramycin resistance gene (aac(3)IV) is flanked by the FRT (FLP recognition target). The required cassettes were generated by PCR using the primer pairs P_novD_apra-tcp830 (5’-AAC CCG GAC CGG TAC GTA CGG CTG AGC TTC CTC GGC TGA ACT AGT GTG TAG GCT GGA GCT GCT TC-3’) and P_novH_apra-tcp830 (5’-CGA CTG ATC AGA AGC TTT GTT CGC ACG TGT GTT GAA CAA TCT AGA CCT CCG ACG TAC GC -3’) for replacement of the region from novD stop codon to novH start codon, and P_novP_apra-tcp830 (5’-ATC GAC CGC GAC GGT GTC TA C TGG CAA CGC ACC CGG TAA ACT AGT GTG TAG GCT GGA GCT GCT TC-3’) and P_novQ_apra-tcp830 (5’- TTC GCG GTC GAA TTC TTG ATT CAT CGG GAG TGC GGG CAT TCT AGA CCT CCG ACG TAC GC-3’) for replacement of the intergenic region novP_novQ. Bold letters represent 39 nt homologous extensions to the DNA regions immediately upstream of novD stop codon and downstream of novH start codon or upstream of novP stop codon and downstream of novQ start codon, including the putative translational stop codons of novD and novP and start codons of novH and novQ, respectively.

The PCR reaction was performed in 50 µl volume with 50 ng template (pMS80 digested with KpnI and SacII), 0.2 mM dNTPs, 50 pmol each primer, 5 % (v/v) DMSO, using the Expand High Fidelity PCR System (Roche Molecular Biochemicals): denaturation at 94 °C for 2 min, then 10 cycles with denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 90 s, then 15 cycles with annealing at 55 °C, and a final elongation step at 72 °C for 5 min. Subsequently, nov-VD6 was modified by excision of aac(3)IV by the FLP recombinase, leaving an in-frame “scar” of 81 nucleotides between the stop codon of novD and tcp830, resulting in cosmid nov-VD7. For construction of nov-VD9, cosmid nov-VD8 was digested with KpnI and SacII and the obtained 4551 bp restriction fragment comprising the region from 671 bp upstream of novN stop codon to 525 bp downstream of novQ start codon, including the apra-tcp830 cassette from pMS80 in exchange with the
intergenic region novP_novQ was introduced into cosmid nov-VD7 via λ-Red-mediated recombination (Gust et al., 2003). *E. coli* XL1 Blue MRF’ cells were transformed with 100 ng DNA and the obtained clones analysed using restriction enzymes and gel electrophoresis. The generated cosmids nov-VD7, nov-VD8 and nov-VD9, carrying the kanamycin resistance gene neo, were then introduced into *S. coelicolor* M512 by PEG-mediated protoplast transformation (Kieser et al., 2000). Kanamycin and apramycin resistant (nov-VD8 and nov-VD9) and kanamycin resistant clones (nov-VD7) were checked for specific genomic integration of the respective cosmids into the ΦC31 attachment site by Southern blot analysis.

**II.10. HPLC Analysis of secondary metabolites**

*S. coelicolor* strains carrying the (modified) novobiocin cluster were cultured in CDM medium as described in section II.6.2.2. After centrifugation, the clear supernatant was analysed by HPLC with a Multosphere RP18-5 column (150 x 4.6 mm; 5 µm; C+S Chromatographie Service, Düren, Germany) with a linear gradient from 60 to 100% methanol in 1% aqueous formic acid and detection at 305 nm. Authentic novobiocin (Fluka) was used as standard.
III. RESULTS

III.1. Investigations on the role of novE in the regulation of novobiocin biosynthesis and its interplay with novG

III.1.1. Inactivation of novE

In order to prove that novE is important for novobiocin biosynthesis, novE was inactivated by an in-frame deletion. For this purpose, novE was replaced in cosmid nov-BG1 with an apramycin resistance cassette flanked by FRT (FLP-recognition target) sites via λ RED-mediated recombination (Datsenko & Wanner, 2000; Gust et al., 2003) resulting in nov-VD1 (Fig. III.1c). The cassette was excised using FLP-recombinase, leaving an in-frame “scar” of 81 nucleotides between the start and stop codon of novE (Fig. III1c). This modified cosmid (named nov-VD2) was subsequently introduced into S. coelicolor M512 by protoplast transformation, and site-specific integration into the genome (Fig. III.1a and b) was confirmed by Southern blot analysis (Fig. III.1d). The deletion of novE was clearly shown by the size of the relevant PstI restriction fragments in comparison to nov-BG1 strains (Fig. III.1d, lane 1 and 2).

HPLC analysis showed that the resulting ΔnovE strains produced novobiocin in a strongly reduced amount (0.7 %) in comparison to S. coelicolor strains carrying the intact novobiocin cluster (Table III.1). Subsequently, novE together with its own putative promoter, i.e. including 599 bp upstream of the start codon of novE, was cloned into the promoterless shuttle vector pWHM3, resulting in pVD4. This plasmid was introduced into the ΔnovE strain S. coelicolor M512 (nov-VD2). Production levels of the resulting transformants reached up to 70 % of those observed in S. coelicolor M512 (nov-BG1) which carries the intact novE gene. This proved that the very low novobiocin production of S. coelicolor M512 (nov-VD2) was indeed due to the inactivation of novE.
Fig. III.1: Inactivation of novE. See next page for details.
(a) Cosmid constructs nov-BG1 and nov-VD2. P = PstI restriction site; T3, T7 = T3 and T7 promoter of the SuperCos1 vector, tet = tetracyclin resistance gene; neo = neomycin/kanamycin resistance gene; int, attP = integrase gene and attachment site of ΦC31. Fragment sizes resulting from digestion with PstI are indicated. Cosmid backbone out of scale. (b) Schematic representation of site specific integration of the cosmids into the genome of Streptomyces coelicolor M512. (c) Schematic presentation of novE replacement and deletion. novE (654bp) was first replaced by an apramycin resistance (aac(3)IV) cassette. Subsequently the cassette was excised using the FLP recombinase, leaving an in-frame “scar” of 81 nucleotides between the start and stop codons of novE. FRT = FLP recognition target. oriT = origin for conjugative transfer. (d) Southern blot analysis. M = Molecular Weight Marker; Lane 1: Streptomyces coelicolor M512 (nov-VD2); lane 2: Streptomyces coelicolor M512 (nov-BG1); lane 3: Streptomyces coelicolor M512. Genomic DNA was digested with PstI. M = Molecular Weight Marker. The entire cosmid nov-BG1 was used as probe.

Table III.1: Influence of novE and novG on novobiocin production in the heterologous host Streptomyces coelicolor M512.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Expression plasmid</th>
<th>Novobiocin (mg/l)</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S.coelicolor M512</td>
<td>control</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S.coelicolor(nov-BG1)</td>
<td>complete cluster</td>
<td>-</td>
<td>19.3</td>
<td>100</td>
</tr>
<tr>
<td>S.coelicolor(nov-BG1)</td>
<td>complete cluster</td>
<td>pWHM3 (empty vector)</td>
<td>20.0</td>
<td>100</td>
</tr>
<tr>
<td>S.coelicolor(nov-VD2)</td>
<td>ΔnovE</td>
<td>pVD4 (novE under control of its genuine promoter)</td>
<td>0.14</td>
<td>0.7</td>
</tr>
<tr>
<td>S.coelicolor(nov-BG1)</td>
<td>complete cluster</td>
<td>pVD4 (novE under control of its genuine promoter)</td>
<td>38.1</td>
<td>191</td>
</tr>
<tr>
<td>S.coelicolor(nov-VD2)</td>
<td>ΔnovE</td>
<td>pVD1 (novG under control of the constitutive ermE* promoter)</td>
<td>59.8</td>
<td>299</td>
</tr>
<tr>
<td>S.coelicolor(nov-VD2)</td>
<td>ΔnovE</td>
<td>pAE8 (novG under control of its genuine promoter)</td>
<td>34.6</td>
<td>173</td>
</tr>
<tr>
<td>S.coelicolor(nov-AE10)</td>
<td>ΔnovG</td>
<td>pWHM3 (empty vector)</td>
<td>0.4</td>
<td>2</td>
</tr>
<tr>
<td>S.coelicolor(nov-AE10)</td>
<td>ΔnovG</td>
<td>pVD4 (novE under control of its genuine promoter)</td>
<td>2.4</td>
<td>12</td>
</tr>
<tr>
<td>S.coelicolor(nov-AE10)</td>
<td>ΔnovG</td>
<td>pAE8 (novG under control of its genuine promoter)</td>
<td>16</td>
<td>80</td>
</tr>
</tbody>
</table>

*Values are means from at least two independent mutants
III. RESULTS

III.1.2. Overexpression of \emph{novE} in \emph{S. coelicolor} M512 (nov-BG1) results in overproduction of novobiocin

Overexpression of pathway-specific activators can result in overproduction of the respective antibiotic (Stutzman-Engwall \emph{et al.}, 1992). Therefore, the \emph{novE} expression plasmid pVD4 was introduced into \emph{S. coelicolor} M512 (nov-BG1) by protoplast transformation. As presented in Table III.1, transformation with pVD4 led to a 1.9-fold increase in novobiocin production in comparison to \emph{S. coelicolor} M512 (nov-BG1) carrying only the empty vector pWHM3.

III.1.3. Complementation of the \emph{novE} mutation by \emph{novG} under control of the constitutive \emph{ermE*} promoter

The results mentioned above are consistent with a role of \emph{novE} as a positive regulator of novobiocin biosynthesis. Previous results (Eustáquio \emph{et al.}, 2005b) had proven that also \emph{novG} acts as a positive regulator of the biosynthesis of this antibiotic. This raised the question whether the two regulators, \emph{novE} and \emph{novG}, act in parallel or in a cascade-like mechanism. In order to investigate the interplay between \emph{novE} and \emph{novG}, a \emph{novG} expression construct was introduced into the \emph{ΔnovE} strain.

Therefore, \emph{novG} was placed under control of the constitutive \emph{ermE*} promoter in the expression vector pUWL201 (Doumith \emph{et al.}, 2000), resulting in pVD1. This plasmid was transformed into the \emph{ΔnovE} strain \emph{S. coelicolor} M512 (nov-VD2) resulting in a dramatic increase of novobiocin production (Table III.1). The fact that novobiocin production in a \emph{ΔnovE} strain can be restored by overexpression of the regulatory gene \emph{novG} strongly indicates that \emph{novE} has a regulatory rather than catalytic function.

III.1.4. Electrophoretic mobility shift assays (EMSA)

A plausible hypothesis to explain the observation described above would be that \emph{novE} is a positive regulator of \emph{novG}, which in turn is a positive regulator of the expression of the biosynthetic enzymes of novobiocin. NovG has been shown to bind to the DNA region between \emph{novG} and \emph{novH} (Eustáquio \emph{et al.}, 2005b). Using gel-mobility shift assays, Alessandra Eustáquio could readily reproduce this result (Fig.III.2a). Subsequently, the coding sequence of \emph{novE} was amplified by PCR and
cloned into the expression vector pRSET B. Using the resulting plasmid pAE18, NovE was expressed in *E. coli* as soluble, N-terminally His$_6$ tagged protein and purified by Ni$^{2+}$ affinity chromatography. This protein was used in band shift assays with three different DNA fragments located upstream of the *novG* start codon. However, no mobility shift was observed. Likewise, no binding could be shown with a DNA fragment located upstream of *novF*, and neither with fragments upstream of *novE* which were tested in order to check for a possible autoregulation of *novE* (Fig.III.2b). These results do not support the hypothesis that *novE* acts by direct DNA binding in the *novEFG* region, and this is in accordance with the fact that bioinformatic analysis does not show any DNA binding motif in the predicted amino acid sequence of NovE. The EMSA mentioned above were carried out by Alessandra Eustáquio.

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Fig. III.2: Electrophoretic mobility shift assays with fragments from the novobiocin biosynthetic gene cluster. See next page for details.
III. RESULTS

Autoradiogram of gel mobility-shift assays using (a) purified His$_6$-tagged NovG; (b) purified His$_6$-tagged NovE. Analysis of DNA-binding activity was carried out as described in (Dangel et al., 2008), using approximately 4 ng of the indicated DIG-end-labelled fragment and either no protein (-) or 1.5 µg of purified His$_6$-tagged NovG or 3 µg of purified His$_6$-tagged NovE protein (+). Above each autoradiogram a map is given showing the location of the DNA fragments used (out of scale; the intergenic regions are oversized in comparison to the coding sequences). The fragments were obtained by digestion of appropriate plasmids or by PCR amplification. For fragments obtained by digestion, the restriction sites are indicated: E, EcoRI; P, PvuI; S, SalI; A, Aval.

III.1.5. Complementation of the novE mutation by novG under control of its own promoter

The failure to show binding of NovE to the novG promoter region questioned the hypothesis that novE acts as positive regulator of novG transcription. Therefore, plasmid pAE8 (Eustáquio et al., 2005b) which contains the structural gene novG together with its genuine promoter region in the promoterless E. coli/Streptomyces shuttle vector pWHM3 (Vara et al., 1989) was used to complement the ΔnovE strain described above. Indeed, complementation was readily achieved with this construct (Table III.1). This proved that novG-expression from its own promoter can occur in the absence of NovE.

III.1.6. Complementation of the novG mutation by novE

A heterologous expression strain carrying a novG deficient novobiocin cluster, i.e. S. coelicolor M512 (nov-AE10) (Eustáquio et al., 2005b), produced only 2 % of the novobiocin amount found in the strains carrying the intact cluster (Table III.1). When this strain was transformed with plasmid pVD4, carrying novE under control of its own promoter, production levels increased approximately 6-fold (Table III.1), but did not reach the original production levels of the strains carrying the intact cluster. In contrast, complementation of the novG-defective strain with an intact copy of novG under control of its own promoter (pAE8) restored most of the original production (Table III.1). Therefore, novG is able to fully complement a novE defect but novE can only partly complement a novG defect.
III.1.7. RT-PCR experiments

In order to investigate whether novG transcription depends on the presence of NovE, and whether novE transcription depends on the presence of NovG, RT-PCR experiments were carried out in the strain carrying the complete cluster, as well as in the strains lacking the novE or the novG gene, respectively (Fig. III.3). The outcome of these experiments showed that at least some novG transcription takes place in the absence of NovE, and likewise that novE transcription occurs in the absence of NovG.

![RT-PCR analysis](image)

**Fig. III.3: RT-PCR analysis.** RT-PCR results of novE and novG transcription in *Streptomyces coelicolor*(nov-BG1) containing the entire novobiocin biosynthetic gene cluster, in the novE-defective strain *Streptomyces coelicolor*(nov-VD2) and in the novG-defective strain *Streptomyces coelicolor*(nov-AE10) (Eustáquio et al., 2005b)); 1-5 = different times of cultivation (1 = 22 hours, 2 = 30 hours, 3 = 44 hours, 4 = 77 hours, 5 = 94 hours); hrdB = house-keeping gene encoding for the sigma factor of the DNA-dependent RNA-polymerase; novE and novG = putative regulatory genes.
III. RESULTS

III.2. Investigations on the genetic organization and transcriptional regulation of the novobiocin biosynthetic gene cluster

III.2.1. Sequence analysis of the novobiocin biosynthetic gene cluster

The novobiocin biosynthetic gene cluster spans 23.4 kb and comprises 20 coding sequences (Li & Heide, 2004; Li & Heide, 2006). The genes novHIJKLM, separated by very short intergenic regions (<19 bp), are responsible for the synthesis and the linkage of the aminocoumarin moiety (Fig. I.3). novQR are responsible for the generation of the prenylated 4-hydroxybenzoate moiety, and novSTUVW for the generation of the deoxysugar. The coding sequences for novQ and novR, as well as of novS and of novV and novW, overlap, suggesting a translational coupling of these genes. novN, novO and novP are responsible for tailoring reactions, i.e. the carbamoylation and methylation of the novobiocin skeleton (Fig. I.3). novF is probably responsible for the availability of 4-hydroxyphenylpyruvate, a precursor of both aromatic moieties of novobiocin (Fig. I.3). gyrBR codes for a resistance gene, and novE and novG for putative regulators of novobiocin biosynthesis (see introduction and III.1.). Large intergenic regions, suggestive of the presence of promoters, are found within the cluster, i.e. upstream of novE (180 bp intergenic region), novG (105 bp), novH (195 bp), novO (230 bp) and gyrBR (376 bp).

For three of these regions, direct or indirect experimental evidence has confirmed their promoter activity, i.e. for the regions upstream novE (Dangel et al., 2008), novH (Eustáquio et al., 2005b) and gyrBR (Thiara & Cundliffe, 1989). In contrast to the streptomycin cluster, which contains four binding-sites of the positive regulator protein StrR, the novobiocin cluster contains only a single binding-site for the StrR-ortholog NovG. This binding-site is situated upstream of novH (Eustáquio et al., 2005b). Both, bioinformatic sequence analysis and electrophoretic mobility shift assays confirmed that no NovG binding-site is situated in the large intergenic region upstream of novO (Alessandra Eustáquio´s thesis).

Therefore, all 16 genes from novH to novW, which are arranged in the same orientation and code for all enzymes of novobiocin biosynthesis from 4 HPP (Fig. I.3),
may be transcribed as a single operon under control of the promoter upstream of \textit{novH}. This promoter is probably regulated by the DNA-binding protein NovG.

### III.2.2. Insertion of transcriptional terminators into the novobiocin biosynthetic gene cluster

A convenient method for the mapping of transcription units is the use of \(\Omega\) (omega) interposon, i.e. a DNA fragment containing an antibiotic resistance marker flanked by short inverted repeats which contain termination signals for transcription (Prentki & Krisch, 1984).

In these studies the \(\Omega\)aac cassette (Blondelet-Rouault \textit{et al.}, 1997) was used which contains the apramycin resistance gene \textit{aacC4}, selectable both in \textit{E. coli} and in \textit{Streptomyces}. While previous studies used conventional cloning techniques to introduce their cassette into the transcription unit of interest (Raynal \textit{et al.}, 2006), in these studies the much more versatile \(\lambda\) RED-mediated recombination technique (Gust \textit{et al.}, 2004) was used to insert the 1.8 kb \(\Omega\)aac cassette into the coding sequence of the genes \textit{novE}, \textit{novF}, \textit{novG}, \textit{novH}, \textit{novO} and \textit{novS}. In each case, the cassette was inserted between nucleotides 3 and 7 of the coding sequence of the gene, i.e. replacing the second codon of the coding sequence. For this purpose, the \(\Omega\)aac cassette was amplified with primers containing 39 bp homolog extensions, identical to the sequences upstream and downstream of the second codon of the gene of interest. The PCR products were used for \(\lambda\) RED-mediated recombination in \textit{E. coli}, using cosmid nov-BG1 as target (Eustáquio \textit{et al.}, 2005a). This cosmid contains the complete novobiocin cluster, as well as the integration functions of the phage \(\Phi\)C31. The resulting cosmids with the inserted \(\Omega\)aac cassettes were integrated into the genome of \textit{Streptomyces coelicolor} M512 as described previously (Eustáquio \textit{et al.}, 2005a). Southern blotting confirmed that in all integration mutants the entire cosmid had integrated site-specifically into the \(\Phi\)C31 attachment site of the genomic DNA (data presented in Johannes Härle’s diploma thesis).
III.2.3. Identification of promoter regions by reverse transcriptase PCR-analysis of termination mutants

As shown previously, heterologous expression of the intact novobiocin cluster in *S. coelicolor* M512 leads to the production of novobiocin, in amounts similar to those formed by the wild-type novobiocin producer strain (Eustáquio *et al.*, 2005a). Correspondingly, reverse transcriptase PCR (RT-PCR) experiments now revealed the presence of transcripts for all genes of the novobiocin gene cluster when the strain was cultivated in novobiocin production medium (Fig. III.4a). Controls without the preceding reverse transcriptase reaction confirmed that the detected signals were due to cDNA rather than to contamination with genomic DNA.

Insertion of the $\Omega$aac cassette into a transcription unit leads to termination of mRNA synthesis, therefore to a lack of RT-PCR signals for the genes downstream of the inserted cassette. Transcription is re-initiated at the next active promoter sequence downstream of the $\Omega$aac insertion. As shown in Fig. III.4b, c and d, insertion of the $\Omega$aac cassette into *novE*, *novF* and *novG* led, as expected, to a complete abolishment of the transcription of the affected genes. In all three cases, however, transcripts of the adjacent gene, i.e. *novF*, *novG* and *novH*, respectively, were detectable, indicating the presence of promoter regions upstream of these genes. In contrast, insertion of $\Omega$aac into *novH* led to a complete abolishment of the transcription of *novHIJKLMNOP*, indicating that all of these genes form a single transcription unit (Fig. III.4e). Transcripts were detected, however, for *novO* and the genes downstream thereof, indicating the presence of a promoter region upstream of *novO*.

Consequently, three additional mutant strains have been generated, carrying $\Omega$aac cassettes in *novO*, *novP* and *novQ* using the same method as described above. In the first two cases, transcripts of the adjacent genes, i.e. *novP* and *novQ* were detected (Fig. III.4f and g), indicating promoter sequences located upstream of these genes. In the strain carrying $\Omega$aac cassette in *novQ*, however, transcription of the following genes was completely abolished, indicating that these genes form a transcription unit (Fig. III.4h). This was confirmed by the results from the strain carrying $\Omega$aac in *novS*, showing complete abolishment of the transcription of *novSTUVW* (Fig. III.4i).
Therefore, our experiments confirmed the previously supplied indirect evidence that a promoter region is located upstream of *novH*, and additionally showed that promoter regions appear to be localised upstream of *novF*, *novG*, *novO*, *novP* and *novQ*. Experimental evidence for the promoter activity of the DNA region upstream of the putative regulator *novE* and the resistance gene *gyrB* has been published previously (Dangel *et al.*, 2008; Thiara & Cundliffe, 1989). Therefore, at least eight promoter regions are present within the novobiocin cluster (Fig. III.4k).
Fig. III.4 See next page for details.
(a-i) Schematic presentation of constructs nov-BG1, nov-JH1 to nov-JH6, nov-JH10 and nov-JH12 including RT-PCR results (the practical part of these investigations was carried out in cooperation with Johannes Härle as diploma student; for details see Johannes Härle’s diploma thesis). (k) Transcriptional organisation of the novobiocin biosynthetic gene cluster including $gyrB^R$.

III.2.4. Real-time PCR investigations of the transcriptional regulation of the novobiocin cluster by novE and novG

In order to investigate the influence of novE and novG on the transcription of the genes of the novobiocin cluster, quantitative RT-PCR (qRT-PCR) experiments using the LightCycler® method were carried out. Suitable primer pairs were chosen for each of the eight genes located downstream of putative promoter sequences within the novobiocin cluster, i.e. novE, novF, novG, novH, novO, novP, novQ and $gyrB^R$. Reaction conditions, i.e. annealing temperature and MgCl$_2$ concentrations, were optimized for each primer pair until a linear relationship between the logarithm of the mRNA concentration and the cycle number was obtained over a concentration range of at least two orders of magnitude.

The respective reaction conditions are described in the experimental, and the PCR primers are shown in Table II.17. For novE, novG and novP the primers designed for the reverse transcriptase PCR could also be used for qRT-PCR. For novF, novH, novO and novQ, however, new primers had to be designed for quantitative mRNA determination. 10 ng of total RNA were used for each LightCycler® reaction. The $hrdB$ transcript, coding for the principle sigma-like transcription factor of *Streptomyces coelicolor*, was used as internal standard and the number of transcripts for each sample was normalized to $hrdB$.

qRT-PCR analysis was then carried out for a strain carrying the intact novobiocin cluster, as well as for strains carrying clusters in which either the gene novE or the gene novG had been inactivated by an in-frame deletion within the coding sequence. The generation of these three strains and the use of *Streptomyces coelicolor* M512 as host for the heterologous expression of the clusters has been described previously (Dangel *et al.*, 2008; Eustáquio *et al.*, 2005a; Eustáquio *et al.*, 2005b).
Cultivation of these three strains in the chemically defined novobiocin production medium was carried out in 24-square deepwell plates (Duetz et al., 2000) which allows much more reproducible cell growth and novobiocin production rates than conventional Erlenmeyer flask cultivations (Stefanie Siebenberg’s thesis).

First, the time course of novobiocin production in this culture system was determined. In accordance with previous results (Stefanie Siebenberg’s thesis) only traces of novobiocin production were detected within the first 48 hours after inoculation, while the highest novobiocin production rate was observed between 72 and 96 hours after inoculation (Fig. III.5a).

In contrast, dry cell weight increases between 24 and 72 hours after inoculation (Stefanie Siebenberg’s thesis). Identical to the observation from the genuine producer strain that novobiocin production starts at the transition from growth phase to stationary phase (Kominek, 1972).

As may be expected, the highest amounts of transcripts for the novobiocin biosynthetic genes novH, novO, novP and novQ were detected immediately before the onset of novobiocin production, i.e. 48 hours after inoculation (Fig. III.5, e-h). All transcripts were still clearly detectable after 72 and 96 hours, i.e. well into stationary phase. Expression of all four genes was perfectly synchronous, which is in accordance with the hypothesis stated above that all these genes may be transcribed as a single operon. For the genes coding for enzymes of novobiocin biosynthesis, i.e. novH and novOPQ, hardly any transcripts were found at 32 hours after inoculation. In contrast, transcripts for the two putative regulators novE and novG could clearly be detected at this time point, indicating that the expression of the regulatory genes may precede the expression of the biosynthetic enzymes (Fig. III.5,b and d). Maximal transcripts amount of novE and novG were found 48 hours after inoculation.

The resistance gene gyrB<sup>R</sup> showed a low level of transcription even at 24 hours after inoculation. A first maximum of gyrB<sup>R</sup> transcripts was detected after 48 hours, i.e. synchronous to the expression of novH and novOPQ. It is tempting to speculate that gyrB<sup>R</sup> may be co-transcribed together with these genes. In contrast to novH and novOPQ, however, the amount of transcripts for gyrB<sup>R</sup> increases again after 72 hours, i.e. when novobiocin concentration is increasing. This is in perfect accordance with
the results of (Thiara & Cundliffe, 1989), who cloned the promoter region of \(gyrB^R\) into a promoter probe vector and showed (by expression in \(Streptomyces lividans\) TK24) that the promoter was induced by cultivation in the presence of novobiocin. Since cultivation in the presence of ciprofloxacin, a gyrase-inhibitor interacting with the A subunit of this enzyme, had the same inducing effect, Thiara and Cundliffe suggested that the induction is not directly mediated by novobiocin but by the change of superhelical density of chromosomal DNA, caused via the gyrase inhibition exerted by novobiocin or ciprofloxacin.

In contrast to the strain with the intact cluster, the two strains with in-frame deletions in the putative regulators \(novE\) and \(novG\) showed only very low novobiocin production (Fig. III.5a). Seven days after inoculation, the strain with the intact cluster had accumulated 45 mg/l novobiocin, whereas the \(\Delta novE\) and \(\Delta novG\) strain had produced less than 0.4 mg/l.

As immediately obvious from Fig. III.5e-h, the amounts of transcripts for the novobiocin biosynthetic genes \(novH\) and \(novOPQ\) were dramatically reduced in the \(\Delta novE\) and \(\Delta novG\) strains in comparison to the strain with the intact cluster, providing for the first time, direct evidence that \(novE\) and \(novG\) act as transcriptional regulators of novobiocin biosynthesis.

Notably, also \(novG\) transcription was strongly reduced in the \(\Delta novE\) strain: at 48 hours after inoculation, the amount of transcripts was only 5% of that observed in the strain with the intact cluster (Fig. III.5d). This suggests that \(novG\) expression is largely, though not entirely dependent on the presence of \(novE\). In contrast, \(novE\) expression was still high in the \(\Delta novG\) strain (Fig. III.5b), suggesting that \(novE\) expression is not dependent on \(novG\).

The resistance gene \(gyrB^R\) shows low basal transcription during the growth phase of both, the \(\Delta novE\) and the \(\Delta novG\) strain, suggesting a low constitutive expression from the \(gyrB^R\) promoter. An alternative explanation is that the basal transcription rate observed for \(gyrB^R\) results in fact from the highly similar constitutively expressed gene \(gyrB\) (=SCO3874) of the host strain \(S. coelicolor\). This gene has 82% identity with \(gyrB^R\) on the nucleotide level, and the priming-site for the forward and reverse
primer for \( gyrb^{R} \) can also be found in this gene, although with five and two mismatches, respectively.

The predicted gene product of \( novF \) shows high sequence similarity to prephenate dehydrogenases and is therefore expected to supply 4-hydroxyphenylpyruvate (4-HPP), the common precursor of both, the prenylated 4-hydroxybenzoate moiety and the aminocoumarin moiety of novobiocin (Fig. I.3). Expression of \( novF \) is detectable at 32 hours after inoculation (Fig. III.5c), i.e. earlier than the expression of the biosynthetic genes \( novH \) and \( novOPQ \). This may ensure the availability of the precursor 4-HPP just before the novobiocin biosynthesis is initiated. Transcription of \( novF \) is remarkably similar to that of \( novE \) in the strain with the intact cluster and in the \( \Delta novG \) strain (Fig. III.5c), which indicates either a close co-regulation or even a co-transcription of both genes.
Fig. III.5: (a) Production curves and (b-i) results of qRT-PCR experiments for *Streptomyces coelicolor* M512 (nov-BG1) containing the entire novobiocin biosynthetic gene cluster, *Streptomyces coelicolor* M512 (nov-VD2) containing the *novE*-defective cluster (Dangel et al., 2008) and *Streptomyces coelicolor*(nov-AE10) containing the *novG*-defective cluster (Eustáquio et al., 2005b).
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III.2.5. Contribution of the promoter regions upstream of \textit{novO}, \textit{novP} and \textit{novQ} to the transcription of the novobiocin cluster

Insertion of the \(\Omega\textit{aac}\) cassette into \textit{novH} led to the complete termination of the transcription of \textit{novHIJKLMN} (see above). Consistently, the strain carrying this insertion (hereafter called \(\Omega\textit{novH}\) strain) did not produce any detectable amounts of novobiocin (<0.2 mg/l), while a strain carrying the intact cluster produced 51 mg/l novobiocin. Notably, however, the \(\Omega\textit{novH}\) strain did also produce detectable amounts of the prenylated 4-hydroxybenzoate moiety (0.3 mg/l). In contrast, a clorobiocin producer strain in which the gene \textit{cloI} (orthologues to \textit{novI}) had been inactivated by in-frame deletion produced 3.75 mg/l of the prenylated 4-hydroxybenzoate moiety (Pojer \textit{et al.}, 2003).

This suggested that in the \(\Omega\textit{novH}\) strain only small amounts of \textit{novQ} and \textit{novR} transcripts (which direct the biosynthesis of prenylated 4-hydroxybenzoate) are formed. This was confirmed by qRT-PCR experiments, composing the expression rate of \textit{novG}, \textit{novH}, \textit{novP} and \textit{novQ} in \textit{S. coelicolor} M512 (nov-BG1) (carrying the intact cluster) and \textit{S. coelicolor} M512 (nov-JH4) (carrying the \(\Omega\textit{novH}\) cluster) (Fig. III.6). In the latter strain the transcription of \textit{novH} was reduced to <1 % in comparison to the former strain, but also the transcription of \textit{novP} and \textit{novQ} was reduced to 3% in comparison to the strain with the intact cluster (Fig. III.6). This can be explained by the hypothesis that transcription of \textit{novO}, \textit{novP} and \textit{novQ} (and of the genes located downstream thereof) is mainly controlled by the \textit{novH} promoter initiating a large transcript from \textit{novH} to \textit{novW}, while the promoter regions upstream of \textit{novO}, \textit{novP} and \textit{novQ} have only minor relevance for the amount of transcripts formed. This is consistent with the results from bioinformatic analysis which showed no NovG binding-site in the \textit{novOPQ} region.
Fig. III.6: Results of qRT-PCR experiments for *Streptomyces coelicolor* M512 (nov-BG1) containing the entire novobiocin biosynthetic gene cluster and *Streptomyces coelicolor* M512 (nov-JH4) containing the Ω<sub>novH</sub>-cluster.
III.2.6. A high novobiocin production is achieved by an optimized novG expression vector

The results described above suggest that control of transcription from the novH promoter is the central mechanism for the regulation of novobiocin biosynthesis. It is tempting to investigate whether this knowledge can be used to further increase novobiocin yields.

In a previous study (Eustáquio et al., 2005b) novobiocin production could be restored in a novG-defective heterologous expression strain by complementation with the multicopy plasmid pAE8, containing an intact copy of novG. Notably, in a strain containing an intact novobiocin cluster, i.e. in S. coelicolor (nov-BG1), pAE8 caused a 2.7-fold overproduction of novobiocin. In the present study, this result could be reproduced, obtaining 2.9-fold overproduction by pAE8 in comparison to the empty vector control.

NovG has been identified as a DNA binding protein. Both, electrophoretic mobility shift assays and bioinformatic sequence analysis have identified the NovG binding-site, a palindromic structure beginning 2 bp downstream of the novG stop codon, i.e. 194 bp upstream of novH start codon (Fig. III.7). Due to the recognition that pAE8, the first plasmid for novG overexpression, contained 135 bp of the intergenic region downstream of novG, i.e. pAE8 included the NovG binding-site (Fig. III.7), it was tempting to speculate that pAE8 may not be an optimal construct for the stimulation of novobiocin production, as the produced NovG protein may bind to the NovG binding-site in pAE8, which is present in many copies in the cell, leaving only a fraction of the produced NovG available for binding to the site in the novobiocin cluster which had been integrated into the genome of the heterologous host. Therefore, a new novG expression plasmid was constructed by Alessandra Eustáquio, named pAE12, which contained just 1 bp of the intergenic region downstream of novG, and therefore did not contain the NovG binding-site (Fig. III.7). Otherwise, this plasmid was identical to pAE8. Transformation of this plasmid into a heterologous expression strain carrying the intact novobiocin cluster resulted in 8.4-fold overproduction of novobiocin compared to the empty vector control. Therefore, transformation with pAE12 turned out to be the most effective method identified so far in order to increase novobiocin production in a heterologous producer strain.
Fig. III.7: Inserts of the *novG* overexpression plasmid pAE8 and pAE12. Both inserts were cloned into the promoterless pWHM3 vector. (Generation of pAE8 and pAE12 were carried out by Alessandra Eustáquio).
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III.3. Regulation of novobiocin production by insertion of a tetracycline-controllable promoter 830 (tcp830)

As presented above, initiation of transcription from the promoter situated upstream of novH, i.e. in the intergenic region novG_novH, is the most important event for the regulation of novobiocin biosynthesis in the heterologous host. In accordance with this finding, the introduction of a strong inducible promoter upstream of novH may, in principle, result in high production and, therefore, may represent a promising strategy to uncouple novobiocin production from its natural regulation cascade.

Till now, the thiostrepton inducible promoter ptipA (Murakami et al., 1989) represents the most widely used inducible promoter for regulating gene expression in Streptomyces spp. providing reliable and controllable gene expression under different circumstances. However, thiostrepton is known to induce a regulon of proteins, and to depend on the presence of an activator, TipAL, and a resistance gene, tsr (Ali et al., 2002; Murakami et al., 1989; Weaden & Dyson, 1998). Therefore, the tetracycline controllable promoter 830 (tcp830) represents an alternative valuable inducible promoter due to its activity in a wide range of natural tetracycline resistant Streptomyces species (Rodriguez-Garcia et al., 2005). Furthermore, using the luxAB genes expressing luciferase as a reporter system, tcp830 has been shown to reach induction factors of up to 270.

The strategy to uncouple novobiocin biosynthesis from its natural regulation cascade, used in this study, was the replacement, not only of the promoter region upstream of novH (Fig. III.4k) but additionally of the entire novEFG region, by the tetracycline controllable promoter 830 (tcp830).

III.3.1. Generation of a novE-novG-double defective mutant

Since the strategy to uncouple novobiocin production from its origin regulation circuit used in this study includes the deletion of both pathway-specific positive regulators novE and novG, a Streptomyces coelicolor M512 strain with a ΔnovEΔnovG-cluster was generated in order to show the effect of inactivation of novE and novG on novobiocin production. Therefore, novE was deleted in cosmid nov-AE10 (ΔnovG),
followed by integration of the resulting $\Delta novE\Delta novG$-cosmid (named nov-VD4) into *S. coelicolor* M512.

For this purpose, *novE* was replaced in cosmid nov-AE10 ($\Delta novG$) by an apramycin resistance cassette flanked by FRT (FLP-recognition target) sites via $\lambda$ RED-mediated recombination (Dat senko & Wanner, 2000; Gust *et al*., 2003), resulting in nov-VD3. This cassette was excised using FLP-recombinase leaving an in-frame “scar” of 81 nucleotides between the start and stop codon of *novE*. The resulting $\Delta novE\Delta novG$-cosmid (named nov-VD4) (Fig. III.8) was subsequently introduced into *S. coelicolor* M512 by protoplast transformation, and its site-specific integration into the genome was confirmed by Southern blot analysis. HPLC analysis showed that inactivation of both pathway-specific regulators, resulted in an almost loss of novobiocin production, i.e. in non detectable amounts. Notably, previous investigations showed that *Streptomyces coelicolor* M512 strains containing either a *novE*- or a *novG*-defective novobiocin biosynthetic gene cluster still produced novobiocin in detectable, but dramatic reduced amounts (0.7% - 2%) in comparison to strains harboring the entire cluster (Table III.1).

Subsequently, *novE* together with its own putative promoter, i.e. including 599 bp upstream of the start codon of *novE*, was cloned into pAE12, that contains *novG* together with its own putative promoter, i.e. 336 bp upstream of the start codon of *novG* in the promoterless shuttle vector pWHM3, resulting in pVD10. The introduction of pVD10 into *S. coelicolor* M512 ($\Delta novE\Delta novG$) led to restoration of novobiocin production in the resulting transformants (120 % in comparision to *S. coelicolor* (nov-BG1, containing the entire novobiocin cluster) and therefore confirmed that the almost loss of novobiocin production in *Streptomyces coelicolor* M512 ($\Delta novE\Delta novG$) was indeed caused by inactivation of both positive regulators *novE* and *novG*.

### III.3.2. Uncoupling of novobiocin production from its natural regulation cascade

In order to increase novobiocin production the apra-tcp830 cassette from pMS80, containing the tetracycline-controllable promoter 830 (tcp830) and an apramycin resistance gene, flanked by FRT-sites (Rodriguez-Garcia *et al*., 2005), was
introduced into cosmid nov-BG1 in exchange with the region from novD stop codon to novH start codon via λ RED-mediated recombination. The resulting cosmid was named nov-VD6 (Fig. III.8). Due to the risk that the effect of tcp830 could not be sufficient to ensure high transcription of all genes from novH to novW, comprising 18 kb, a second tcp830 cassette was introduced into the novobiocin biosynthetic gene cluster in exchange with the intergenic region novP_novQ. For this purpose, the apramycin resistance gene in nov-VD6 was firstly deleted by expression of the FLP-recombinase, leaving a “scar” of 81 nucleotides upstream of tcp830. The resulting cosmid was named nov-VD7 (Fig. III.8). The following attempt to introduce a second apra-tcp830 cassette into nov-VD7 in exchange with the intergenic region novP_novQ, by the repeated use of λ RED-mediated recombination did not succeed due to sequence homologies between the newly generated PCR-product and the already existing tcp830 sequence situated upstream of novH. Instead, a complete loss of the region from novH start codon to novQ start codon was observed. For this reason, λ RED-mediated recombination was modified as follows: the first step was the introduction of the apra-tcp830 cassette from pMS80 into cosmid nov-BG1 in exchange with the intergenic region novP_novQ via λ RED-mediated recombination, resulting in cosmid nov-VD8 (Fig. III.8). After digestion of nov-VD8 with KpnI and SacII, the obtained 4551 bp restriction fragment, comprising the region from 671 bp upstream of novN stop codon to 525 bp downstream of novQ start codon, including the apra-tcp830 cassette within the intergenic region novP_novQ, was successfully introduced into cosmid nov-VD7 via λ RED-mediated recombination, resulting in cosmid nov-VD9 (Fig. III.8). No illigiteme recombination was observed this time due to the long flanking DNA sequences for homologous recombination by using the 4551 bp restriction fragment instead of the 1596 bp PCR product. Subsequently, the obtained cosmids nov-VD7, nov-VD8 and nov-VD9 were introduced into Streptomyces coelicolor M512 by protoplast transformation, followed by confirmation of their site-specific integration into the genome by southern blot analysis.
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Fig. III.8: Schematic presentation of constructs nov-BG1, nov-VD4, nov-VD6, nov-VD7, nov-VD8 and nov-VD9.

III.3.3. Optimization of induction-conditions for tcp830 towards maximal novobiocin production

Investigations on the determination of optimal aTc (anhydro-Tetracycline)-concentration and time for induction of tcp830 towards maximal novobiocin production were carried out using one transformand of *S. coelicolor* M512 (nov-VD9) and *S. coelicolor* M512 (nov-BG1), harboring the entire novobiocin biosynthetic gene cluster, as a control.

For this purpose, cultivation was carried out in absence and after addition of 0.25, 0.5, 1, 2, 4, 8 or 16 µg/ml aTc at inoculation time. HPLC-analysis confirmed that *S. coelicolor* M512 (nov-VD9) produced novobiocin even in absence of aTc, but only in reduced amounts (24%) in comparison to *S. coelicolor* M512 (nov-BG1) harboring the entire novobiocin biosynthetic gene cluster. In contrast, the induction of tcp830 in
S. coelicolor M512 (nov-VD9) by addition of 0.25 to 2 µg/ml aTc resulted in a dramatic increase of novobiocin production (20-fold), representing not only restoration in comparison to S. coelicolor M512 (ΔnovEΔnovG), but additionally an up to 4.9-fold overproduction of novobiocin in comparison to S. coelicolor M512 (nov-BG1), harboring the entire novobiocin biosynthetic gene cluster under its natural regulation (Fig. III.9a). In contrast, addition of 0.25 to 2 µg/ml aTc did not significantly influenced novobiocin production in S. coelicolor M512 (nov-BG1) (Fig. III.9a). Further investigations on dry weight showed, that addition of aTc in concentrations from 0.25 to 2 µg/ml exhibits almost no effect on S. coelicolor M512 (nov-BG1) and only small positive effects on S. coelicolor M512 (nov-VD9) (Fig. III.9b). In accordance to these results, the addition of 1 µg/ml aTc for induction of tcp830 was determined to achieve maximal novobiocin production.

Subsequently, the optimal time for induction of tcp830 was investigated by addition of 1 µg/ml aTc at inoculation-time and after 6, 12, 24, 36, 48 or 72 hours of cultivation. HPLC-analysis showed that modification of induction-time from inoculation-time did not lead to a further increase of novobiocin production (data not shown). Therefore, addition of 1 µg/ml aTc at inoculation time was used for further investigations.
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Fig. III.9 (a) Novobiocin production and (b) dry weight of S.coelicolor M512 (nov-BG1), containing the entire novobiocin cluster and S.coelicolor M512 (nov-VD9), containing nov-BG1 with the region from novD stop codon to novH start codon and novP stop codon to novQ start codon replaced with (apra-)tcp830 in absence and after addition of 0.25, 0.5, 1, 2, 4, 8 and 16 mg/l aTc at inoculation time.

III.3.4. Quantitative comparison of novobiocin production under natural promotor and inducible promotor control

For quantitative analysis of novobiocin production, three independent transformants of S.coelicolor M512 (nov-VD7), S.coelicolor M512 (nov-VD8), S.coelicolor M512 (nov-VD9) and S. coeliocolor M512 (nov-BG1) as a control were cultivated in absence and after addition of 1 µg/ml aTc at inoculation-time. The following HPLC-analysis of secondary metabolite production showed, that all mutants harboring tcp830, produced novobiocin even in absence of aTc, but only in reduced amounts (12 to 48%) in comparison to S.coelicolor M512 (nov-BG1) (data not shown).
However, the induction of tcp830 led to a dramatic increase of novobiocin production in all three mutants, resulting in overproduction of novobiocin in comparison to \textit{S. coelicolor} M512 (nov-BG1), i.e. 1.5-fold for \textit{S. coelicolor} M512 (nov-VD8), 1.6-fold for \textit{S. coelicolor} M512 (nov-VD9) and 2-fold for \textit{S. coelicolor} M512 (nov-VD7) (Fig. III.10). In accordance with these results, introduction of tcp830 upstream of novH, i.e. in \textit{S. coelicolor} M512 (nov-VD7), has been confirmed of being sufficient not only to fully complement the novEFG-deletion, but moreover to overexpress the entire novobiocin biosynthetic gene cluster, resulting in overproduction of novobiocin. Quantitative HPLC-analysis of Ring A formation showed, that \textit{S. coelicolor} M512 (nov-VD7) and \textit{S. coelicolor} M512 (nov-VD9) did not produce any detectable amounts of Ring A. In contrast, the introduction of tcp830 in exchange with the intergenic region novP_novQ, i.e. in \textit{S. coelicolor} M512 (nov-VD8), resulted in a 4.2-fold overproduction of Ring A in comparison to \textit{S. coelicolor} M512 (nov-BG1) (Fig. III.10). This observation is in accordance with the localization of tcp830 in cosmid nov-VD8 (Fig. III.8), i.e. upstream of novQ, that in addition to novR encodes enzymes involved in Ring A formation (Li & Heide, 2004).

![Graph](image)

**Fig. III.10:** See next page for details.
Novobiocin production (black bars) of *S. coelicolor* M512 (nov-BG1) containing the entire novobiocin biosynthetic gene cluster, *S. coelicolor* M512 (nov-VD7) containing nov-BG1 with the region *novD* stop codon to *novH* start codon replaced by tcp830, *S. coelicolor* M512 (nov-VD8) containing nov-BG1 with the region *novP* stop codon to *novQ* start codon replaced by apra-tcp830 and *S. coelicolor* M512 (nov-VD9) containing nov-VD7 with the region *novP* stop codon to *novQ* start codon replaced by apra-tcp830 and Ring A formation (white bars) of *S. coelicolor* M512 (nov-BG1) and *S. coelicolor* M512 (nov-VD8). Values are means of at least three independent mutants and two independent cultivations.
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IV.1. Investigations on the role of novE in the regulation of novobiocin biosynthesis and its interplay with novG

The biosynthetic gene cluster of the aminocoumarin antibiotics novobiocin, clorobiocin and coumermycin A₁ each contain two putative regulatory genes with high similarity within the clusters, i.e. \textit{novG/cloG/couG} and \textit{novE/cloE/couE}. The function of NovG as a DNA binding protein and positive regulator of novobiocin biosynthesis has been established (Eustáquio \textit{et al.}, 2005b). This study investigated the function of \textit{novE}.

The observations that the inactivation of \textit{novE} led to a strong reduction but not to a complete abolishment of novobiocin production, that the overexpression of \textit{novE} led to an increase of novobiocin production and that the \textit{novE} defect could be complemented by an overexpression of the regulatory gene \textit{novG} supported the hypothesis that \textit{novE} had a regulatory rather than a catalytic function.

It was tempting to speculate that NovE may regulate the transcription of \textit{novG}. However, RT-PCR experiments suggested that at least some \textit{novG} transcription can occur in the absence of NovE, and that \textit{novE} transcription can occur in the absence of NovG. Correspondingly, overexpression of \textit{novG} under control of its own promoter stimulated novobiocin production even in a \textit{novE}-defective strain. Vice versa, a \textit{novG} defect strain could be complemented (at least partially) by overexpression of \textit{novE}.

These studies were carried out by heterologous expression of the modified cluster in the completely sequenced host \textit{S. coelicolor}. The genome of \textit{S. coelicolor} (Bentley \textit{et al.}, 2002) does not contain any orthologues of \textit{novE} or \textit{novG}, which may functionally replace the experimentally deleted genes within the novobiocin cluster.

Taken together the results of these investigations prove that both \textit{novE} and \textit{novG} act as positive regulators of novobiocin biosynthesis, although the exact mechanism of their interplay was not clarified by these studies. Notably, NovE has been identified as a newly discovered tool to enhance novobiocin production. It remains to be shown whether the nine orthologues of \textit{novE} deposited in the database, among them \textit{lmbU}...
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from the lincomycin and rubC4 from the rubradirin biosynthetic gene cluster, can likewise be used to increase the production of the respective secondary metabolites.

IV.2. Genetic organization and transcriptional regulation of the novobiocin biosynthetic gene cluster

In order to investigate whether novE and novG act as transcriptional activators of novobiocin biosynthetic genes and to investigate the interplay of these two regulators, qRT-PCR experiments have been carried out.

After the establishment of qRT-PCR for investigations on the novobiocin cluster, the following experiments provide the first direct proof that novE and novG act as transcriptional regulators of novobiocin biosynthesis. For novG, this result was expected, as its gene product shows 41% identity on the amino acid level to strR, an established transcriptional regulator of streptomycin biosynthesis (Retzlaff & Distler, 1995; Tomono et al., 2005). In contrast, novE has only few orthologues in other secondary metabolic gene clusters and sequence genomes, and no previous evidence existed on the function of these genes.

qRT-PCR experiments, comparing transcription levels in strains containing either an intact or a novG-defective novobiocin cluster, showed that effective transcription from the novH promoter depended on the presence of novG (Fig. III.5e). At least the seven genes novHIJKLMN are apparently transcribed as a single operon (Fig. III.4e). In a mutant containing a terminator cassette (Ωaac) in the coding sequence of either novH, novO or novP, RT-PCR showed transcripts of novO, novP and novQ, respectively, suggesting the presence of promoters upstream of these genes. However, qRT-PCR with the ΩnovH mutant proved that transcription of novQ was much lower in this mutant than in a strain with the intact cluster, showing that the promoters upstream of novO, novP and novQ contributed only little to the overall transcription of novQ. The most plausible explanation of this observation is that the rate of transcription of novOPQ is primarily controlled by the novH promoter which initiates transcription of a large polycistronic mRNA. Since also novQRSTUWW apparently are part of a single operon (Fig. III.4h and i), it appears likely that all of the 16 genes from novH to novW, which together direct all steps of novobiocin
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biosynthesis from glucose-1-phosphate and 4-hydroxyphenylpyruvate or tyrosine (Fig. I.3), are transcribed predominantly in form of a single 18,000 nt transcript. Possibly, even the resistance gene $gyrB^R$ may be included in this transcript, extending it to 20,000 nt. Transcripts of this size are not unusual in secondary metabolic gene clusters, as the coding sequences of modular polyketide synthase or nonribosomal peptide synthase genes often span even larger DNA regions. However, other examples of secondary metabolic gene clusters where 15 or 16 individual genes are transcribed onto a single mRNA have not been described yet.

Within the suggested large operon starting with $novH$, RT-PCR had indicated the presence of internal promoters upstream of $novO$, $novP$ and $novQ$. Internal promoters within operons have been described previously in *Streptomyces*, e.g. galP2, a low-level constitutive promoter internal to the galactose operon in *Streptomyces lividans* and *Streptomyces coelicolor* A3(2).

A recent study suggested that in *Streptomyces coelicolor* (in contrast to *E. coli* and *Bacillus subtilis*), expression levels of the individual genes of an operon decrease with increasing distance from the transcription start, and suggested that the frequently encountered internal promoters may ensure adequate transcription of the terminal genes of an operon (Laing et al., 2006). Under experimental conditions, however, transcription from the internal promoters upstream of $novO$, $novP$ and $novQ$ was low, and it cannot be decided whether they have any significant role in the transcription of the novobiocin biosynthetic genes.

Sequence analysis did not show the characteristic sequence of NovG binding-site in the $novOPQ$-region, and EMSA assays did not show binding NovG (or NovE) in the intergenic region upstream of $novO$. This, and the low transcript levels for $novQ$ in the $\Omega novH$ strain suggest, that the weak internal promoters in the $novOPQ$ region are not regulated by $novG$ or $novE$, and may not be regulated at all.

This study shows that effective transcription of $novG$ depends on the presence of $novE$ (Fig. III.5d). This suggests a cascade-like regulation mechanism of $novE$ and $novG$, i.e. $novE$ triggers transcription of $novG$, which in turn triggers transcription of the novobiocin biosynthetic genes. Consistent with this hypothesis, it was shown that novobiocin formation in a $novE$-defective mutant could be restored by an intact copy
of novG on a multicopy plasmid, while novobiocin formation in a novG-defective mutant remained low even after expression of novE from a multicopy plasmid.

Transcription of the novobiocin resistance gene gyrB\(^R\) may initially occur as part of the large transcript starting from novH, but later on obviously follows a different pattern (Fig. III.5i). Our results may be perfectly explained by the hypothesis of (Thiara & Cundliffe, 1989) that a promoter located upstream of gyrB\(^R\) is regulated by the superhelical density of DNA, which in turn is influenced by the accumulation of the gyrase inhibitor novobiocin, acting on the constitutively expressed aminocoumarin-sensitive gyrB\(^S\) subunit of gyrase.

Therefore, the results of the study on the genetic organisation and transcriptional regulation of the novobiocin cluster can be summarized in the model depicted in Fig. IV. In this model, novE positively regulated transcription of novG. Since we could not demonstrate binding of the NovE protein to the DNA region upstream of novG (Fig. III.2b), the mechanism of regulation by novE is yet unknown. NovE may undergo a modification (e.g. phosphorylation) and bind to another, unknown protein before interacting with the novG promoter region, or it may release a repressor from this region, or trigger another event which ultimately induces transcription from the novG promoter.

Expression of the novG-ortholog strR in the streptomycin cluster is triggered by the binding of AdpA to defined binding-sites located in the 406 bp intergenic region upstream of strR (Tomono et al., 2005). However, the consensus sequence for the AdpA binding-site is not found in the 105 bp intergenic region upstream of novG, and neither in the coding sequence of the preceding gene novF. Therefore, regulation of the novG expression appears to be different from that of strR expression in the streptomycin cluster.

All experiments of this study were carried out in a heterologous producer strain S. coelicolor M512 (a derivative of S. coelicolor A3(2)) with the novobiocin gene cluster integrated into the ΦC31 attachment site of the chromosome. Exactly as described for the genuine producer strain (Kominek, 1972) novobiocin production in the CDM medium (see experimental) occurred after the growth phase, suggesting that novE and novG expression may be regulated in a similar timely fashion as in the genuine
novobiocin producer strain. The exact mechanism for this regulation remains to be elucidated in both strains.

Clearly, this study suggests that initiation of transcription from the novH promoter is the most important event for the regulation of novobiocin biosynthesis in the heterologous host. Transcription from this promoter is controlled by the DNA-binding protein NovG, which binds to a well-defined inverted repeat sequence in the intergenic region between novG and novH. (Fig. IV). This knowledge could be utilized to achieve an 8.4-fold overproduction of novobiocin by utilization of an optimized expression vector for novG.

Fig. IV: Model of the genetic organization and transcriptional regulation of the novobiocin biosynthetic gene cluster.
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IV.3. Regulation of novobiocin production by tetracycline-controllable promoter 830 (tcp830)

Antibiotic production is regulated by both, pathway-specific and global regulatory genes (Takano, 2006). Since the borders of the novobiocin biosynthetic gene cluster have been identified (Dangel et al., 2008; Eustáquio et al., 2005a) and the functions of most of the genes contained therein has been (Li & Heide, 2004), the presence of further pathway-specific regulators, in addition to novE and novG, may be excluded. Inactivation of both regulators, i.e. novE and novG, resulted in an almost loss of novobiocin production.

Strong promoters were commonly used to overexpress single genes, since their use to express entire gene clusters is often limited by the fact that gene clusters consist of multiple transcripts. Recently, the replacement of pathway-specific regulatory genes and native promoter regions within a biosynthetic gene cluster with ermEp*, has been described as a strategy not only to uncouple secondary metabolite production from its natural regulation cascade, but moreover to result in enhanced antibiotic production, i.e. the replacement of the 3.4 kb regulatory region in the jadomycin biosynthetic gene cluster, including four regulatory genes and the promoter region PJ, by the constitutive promoter ermEp* resulted in increased jadomycin B production in Streptomyces venezuelae (Zheng et al., 2007).

In addition to ermEp*, representing a constitutive promoter, several inducible promoters are available. Previous investigations confirmed the tetracycline controllable promoter 830 (tcp830) to reach induction factors of up to 270 (Rodriguez-Garcia et al., 2005). In the present study tcp830 has been confirmed of being sufficient to transcribe an entire gene cluster of at least 18 kb, resulting in high antibiotic production. Notably, a recent study (Laing et al., 2006) suggested that in Streptomyces coelicolor (in contrast to E. coli and Bacillus subtilis), expression levels of the individual genes of an operon decreases with increasing distance from the transcription start.

The finding that the replacement of the entire novEFG-region, including the promoter region upstream of novH, by tcp830, i.e. in S.coelicolor M512 (nov-VD7), resulted in 2-fold overproduction of novobiocin is in accordance with the result obtained in this
thesis that initiation of transcription from the \textit{novH} promoter is the most important event for the regulation of novobiocin biosynthesis in the heterologous host.

Moreover, the observed high novobiocin production in \textit{S. coelicolor} M512 (nov-VD7) and \textit{S. coelicolor} M512 (nov-VD9), both lacking the putative prephenate-dehydrogenase \textit{novF}, excludes an essential function for \textit{novF} in novobiocin biosynthesis in the heterologous host. \textit{novF} may be functionally replaced by SCO1761, encoding for a putative prephenate-dehydrogenase with 44\% sequence homology on the amino acid level to \textit{novF}.

In conclusion, the control of novobiocin production by use of the inducible promoter \textit{tcp830} has been identified as a new tool to enhance antibiotic production.
V. REFERENCES


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